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CLINICAL AND EXPERIMENTAL

CIRRHOSIS OF THE LIVER

IMPENDING HEPATIC COMA AND INCREASED BLOOD AMMONIUM CONCENTRATIONS DURING PROTEIN HYDROLYSATE INFUSION

LESLIE T. WEBSTER, JR., M.D.,* AND CHARLES S. DAVIDSON, M.D.
BOSTON, MASS.

INTRODUCTION

THE ingestion of protein by susceptible patients with hepatic cirrhosis or Eck's fistula may induce impending hepatic coma associated with increased venous blood ammonium concentrations.[1, 4] Parenteral administrations of protein or amino acids, however, has been little studied and, according to one report, did not alter the clinical course of patients with hepatic coma.[5]

In the present study, protein hydrolysates were given intravenously to patients with hepatic cirrhosis in order to provide protein precursors while simultaneously avoiding the complication of impending hepatic coma (mental confusion and "flapping" tremor).[6] Although these protein hydrolysates were well tolerated at slow infusion rates, their rapid intravenous administration to patients with hepatic cirrhosis produced impending hepatic coma associated with marked increases in venous blood ammonium concentrations. Further studies indicated that ammonium both contained in the hydrolysate and produced by its administration could account for the increased blood ammonium concentrations observed.

From the Thorndike Memorial Laboratory and Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.

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*Present Address: City Hospital, Scranton Road, Cleveland 9, Ohio.

MATERIALS AND METHODS

Five men ranging in age from 41 to 66 years were studied on the ward of the Thorndike Memorial Laboratory. All patients had clinical diagnoses of hepatic cirrhosis and histories of excessive alcoholic and poor dietary intakes; the clinical diagnosis was confirmed by liver biopsy in one patient (P. D., Table I). On hospital admission or shortly thereafter, 3 patients had evidence of impending hepatic coma; all 5 patients improved during their hospitalizations. At the time protein hydrolysate infusions were given, none of these patients was confused or tremulous, nor did any display evidence of upper gastrointestinal hemorrhage or shock. Serum nonprotein nitrogen concentrations were below 30 mg. per 100 ml. in every instance.

TABLE I. CLINICAL AND LABORATORY DATA OF FIVE MEN WITH CIRRHOSIS

PATIENT, AGE	PHYSICAL EXAMINATION*	BSP†	SERUM BILIRUBIN (MG. PER 100 ML.)	COMMENT
J. S. 44	Icterus, ascites, edema	—	4.5	I.C.‡ and marked icterus on hospital admission
J. A. 66	Ascites, edema	44	2.5	I.C.‡ and icterus on hospital admission
J. C. 41	Splenomegaly	24	1.2	History of ascites and edema
J. T. 50	Edema	33	0.9	Delirium tremens and I.C. on admission; history of ascites
P. D. 45	Edema	12	1.6	Icterus on admission

*All patients had hepatomegaly and spider angioma.

†BSP = Per cent of Bromsulphalein retained in serum 45 minutes after injection of dye (5 mg. per kilogram of body weight).

‡I.C. = Impending hepatic coma.

Except for J. C., patients received weighed diets daily which contained from 50 to 90 Gm. of protein, 200 to 2600 calories, and approximately 200 mg. of sodium. The diet provided J. C. was similar to the above except that it was unrestricted in sodium content. Nitrogen-containing foods or medications were not given for at least 12 hours before each series of observations. Otherwise, the nitrogen content of the diets was not changed.

Ammonium chloride, glycine,* DL-alanine,* L-glutamic acid,* L-aspartic acid,* and protein hydrolysates were administered intravenously, usually by constant rate infusion apparatus. The protein hydrolysate solutions given were commercially available (Table II).† Ammonium chloride and amino acids were weighed out and either made up to a given volume with sterile 5 per cent dextrose and water or added to a protein hydrolysate solution. All administered solutions were analyzed for total[7] and ammonium nitrogen.

McDermott and Adams'[2] modification of the Conway technique was used to determine ammonium nitrogen (NH_4-N)‡ in blood and in the nitrogenous substances administered. This method employs a 10-minute diffusion time and results, calculated for blood as recommended by Conway, were expressed as micrograms of total NH_4-N per 100 ml. for blood or as milligrams of total NH_4-N per milliliter for the nitrogenous solutions employed. Antecubital venous blood, obtained in a heparinized§ syringe, was immediately introduced into Conway units and was analyzed in duplicate for NH_4-N at least once before, and every 15

*Provided through the courtesy of Dr. Augustus Gibson of Merck, Sharpe & Dohme, Rahway, N. J.

†The Aminosol was provided through the courtesy of Dr. Douglas Frost, Abbott Laboratories, North Chicago, Ill.

‡It is not known whether ammonium exists free in the blood or whether it is formed only after blood is shed.

§The heparin solution employed was Liquaemin, from Organon, Inc., Orange, N. J. The use of a heparinized syringe did not change the blood NH_4-N concentration.

minutes during, each infusion. Determinations on 2 successive blood samples drawn during a day in the same fasting* patient (12 patients with cirrhosis, 67 determinations) were reproducible to within 7 mcg. per 100 ml.

The per cent retention of intravenously administered Bromsulphalein dye (5 mg. per kilogram of body weight) was measured in serum samples obtained 45 minutes after dye injection and the serum bilirubin concentrations reported were performed by the standard technique.[8]

RESULTS

Impending Hepatic Coma and Increased Blood Ammonium Concentrations With Rapid Rate Infusions of Protein Hydrolysates.—Patient J. S. received rapid intravenous infusions (9.2 and 15.4 ml. per minute) of 2 protein hydrolysates on separate occasions (Fig. 1). On the first occasion, he demonstrated mental confusion, a "flapping" tremor, and increasing blood NH_4-N

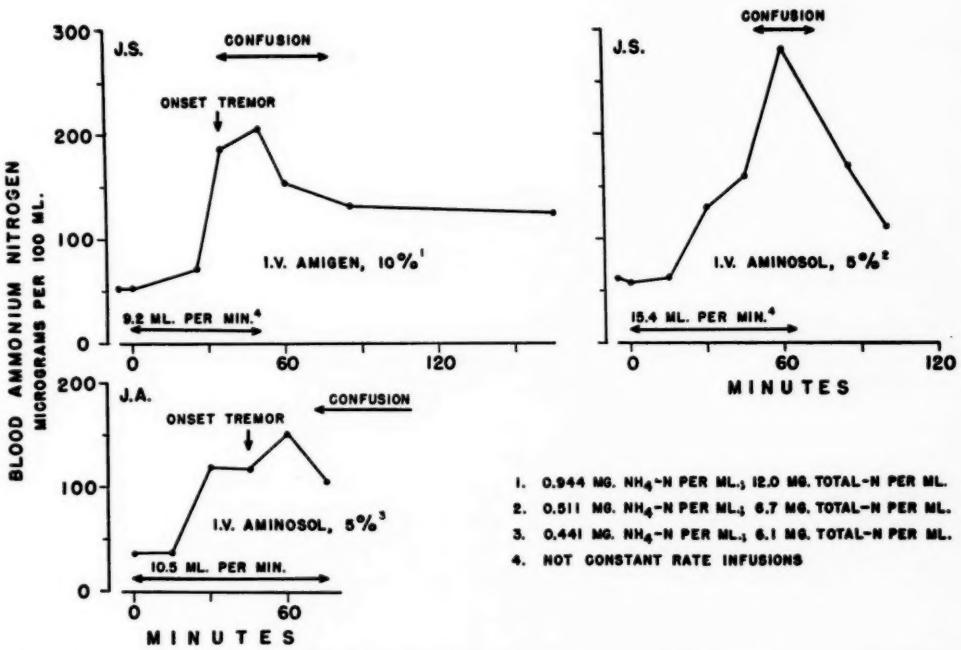


Fig. 1.—Impending hepatic coma and increased blood ammonium in 2 cirrhotic patients during intravenous protein hydrolysates.

concentrations with an infusion of 10 per cent Amigen. An infusion of another protein hydrolysate, 5 per cent Aminosol, given to this clinically improving patient 24 days later, induced less confusion and failed to induce a tremor despite a higher rise in venous blood NH_4-N concentrations. Patient J. A. also developed mental confusion, a "flapping" tremor, and increased venous blood NH_4-N concentrations during a rapid rate (10.5 ml. per minute) infusion of 5 per cent Aminosol (Fig. 1). The occurrence of confusion,

*Fasting is defined here as not having eaten food containing nitrogen for at least 12 hours.

"flapping" tremor and elevated venous blood NH_4-N concentrations in these patients suggested that protein hydrolysates infused rapidly were capable of precipitating impending hepatic coma.

Ammonium Content of Protein Hydrolysates.—Because ammonium salts may induce impending hepatic coma in patients with hepatic disease,[9] ammonium nitrogen was analyzed in each of 4 protein hydrolysates (Table II). Ammonium-nitrogen composed from 3.2 per cent (Travamin) to 7.5 per cent (average present in Aminosol) of the total nitrogen present or ranged from 0.25 to 0.94 mg. per milliliter of hydrolysate solution.

TABLE II. TOTAL-N AND AMMONIUM-N CONTENT OF 4 PROTEIN HYDROLYSATES

NAME OF HYDROLYSATE; COMPANY	TYPE	AVERAGE TOTAL NITROGEN (MG. PER ML.)	AVERAGE AMMONIUM NITROGEN (MG. PER ML.)
Amigen 10 per cent Mead Johnson & Company Evansville, Ill.	Pancreatic hydrolysate of casein	12.5 (3)*	0.876 (5)* Range 0.814-0.944
Travamin 5 per cent Baxter Laboratories, Inc. Morton Grove, Ill.	Enzymatic hydrolysate of bovine plasma	7.7 (1)*	0.250 (1)*
Cutter 5 per cent protein hydrolysate in 5 per cent dextrose Cutter Laboratories Berkeley, Calif.	Pancreatic hydrolysate of casein	6.0 (1)*	0.280 (2)* Range 0.276-0.284
Aminosol 5 per cent Abbott Laboratories Chicago, Ill.	Acid hydrolysate of beef blood fibrin	6.2 (3)*	0.465 (7)* Range 0.395-0.521

*Numbers in parentheses represent number of samples analyzed.

Blood Ammonium Concentrations With Slow Rate Protein Hydrolysate Infusions: Possible Deamination of Infused Amino Acids.—The ammonium found by analysis in the hydrolysate might have caused the elevated blood NH_4-N levels observed in the 2 patients given protein hydrolysates rapidly. This hypothesis was tested by giving slower infusions of a protein hydrolysate (5 per cent Aminosol), 2 amino acids, and ammonium chloride to 3 additional patients with cirrhosis (Table III, Figs. 2 and 3).

Slow hydrolysate infusion produced a delayed increase in blood NH_4-N concentration 60 to 75 minutes after starting (the Aminosol infusions, Figs. 2 and 3). When the infusion rate was increased approximately fivefold (in Patient P. D.), a prompt increase resulted (Fig. 3 and 2 examples in Table III). Thus, a prompt blood NH_4-N increase occurred only when this protein hydrolysate was infused at a sufficiently rapid rate. When the hydrolysate was infused slowly, the delayed blood NH_4-N increase observed did not result from free ammonium analyzed in the protein hydrolysate since the same or a lesser amount of ammonium, given intravenously as ammonium chloride, effected a prompt rise in venous blood NH_4-N concentrations (Figs. 2 and 3).

Slow rate infusions of DL-alanine (Figs. 2 and 3) and glycine (Table III, Patient J. C.) produced delayed blood NH_4-N increases, similar to those observed with slow rate infusions of the protein hydrolysate (Patient J. C., Table

III). These individual amino acid solutions contained about the same total nitrogen but far less ammonium nitrogen than the protein hydrolysate (Table III). Quantities of ammonium similar to those analyzed in the infused solutions of alanine did not produce a significant rise in blood NH_4-N concentrations when infused as ammonium chloride (Table III). These observations suggested that the delayed increases in blood NH_4-N concentration observed with slow rate infusions of both protein hydrolysate and alanine might be produced by the deamination of infused amino acids.

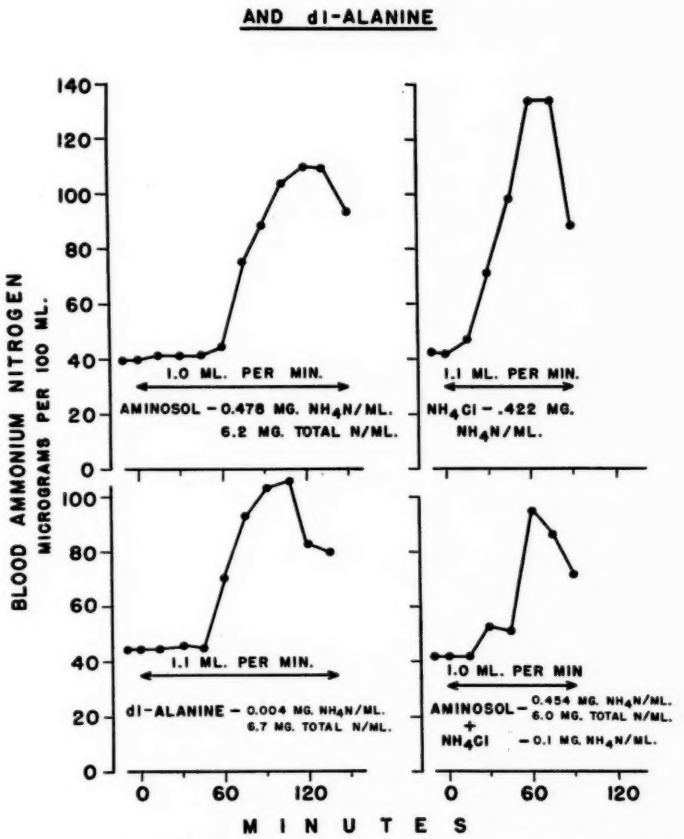


Fig. 2.—Venous blood ammonium in cirrhotic Patient J. T. during infusions of 5 per cent Aminosol, ammonium chloride, and DL-alanine.

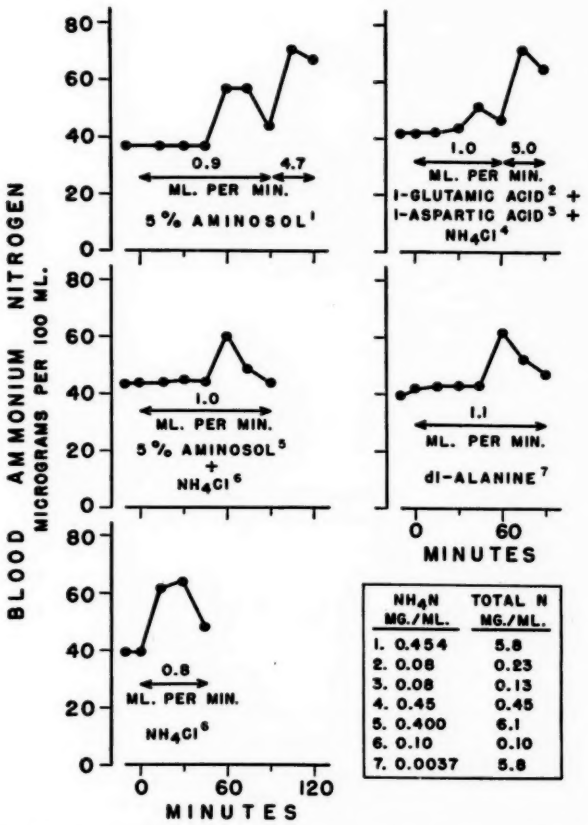
The Lack of a Prompt Blood Ammonium Increase With Slow Rate Protein Hydrolysate Infusions; the In Vivo "Neutralization" of Infused Ammonium.—Slow rate infusions of a protein hydrolysate did not produce prompt rises in blood NH_4-N concentrations, although infusions of ammonium chloride containing the same or lesser amounts of ammonium than the hydrolysate produced prompt increases (Figs. 2 and 3). In Patient J. T., different quantities of ammonium chloride were added to 2 solutions of the same protein hydrolysate. The resulting solutions, when infused slowly, produced prompt as well as

TABLE III. EFFECT OF SLOWLY INFUSED NITROGENOUS SUBSTANCES ON VENOUS BLOOD NH_4-N CONCENTRATIONS IN 3 PATIENTS WITH HEPATIC CIRRHOSIS

PATIENT	DATE	SOLUTION GIVEN*	ANALYZED NH_4-N (MG. PER ML.)	TOTAL NH_4-N ADMINISTERED (MG. PER MIN.)	BLOOD NH_4-N CONCENTRATIONS (MCG. PER 100 ML.) (MINUTES) 0	15	30	45	60	75	90	105	120	135	150
J. T.	2/24	Aminosol 5%	0.48	0.48	41,41	42	41	42	44	76	89	103	115	114	94
	2/15	NH_4Cl†	0.42	0.46	43,42	47	71	98	134	134	89				
	2/17	DL-alanine 4.2%	0.0039	0.0043	46,46	46	47	46	71	93	103	106	83	80	
	2/22	NH_4Cl†	0.004	0.0038	52,52	54	54	59	55	56					
				0.0070							58	58	57		
	3/3	Aminosol 5%	0.47												
		and	and	0.97	48,48	58	91	85	102	102	87				
		NH_4Cl	0.50												
	3/4	Aminosol 5%	0.45												
		and	and	0.55	42,43	42	53	51	95	87	72				
		NH_4Cl	0.10												
P. D.	4/1	Aminosol 5%	0.45	0.41	37	36	36	36	57	57	44				
				2.12								71	67		
	3/30	Aminosol 5%	0.45	2.25	40,42	82	82	60	48						
	4/8	DL-alanine 3.7%	0.0037	0.0041	40,42	42	43	43	62	53	48				
	4/11	NH_4Cl†	0.0038	0.0038	42,42	42	43	42	44						
				0.020						54	43	42			
	5/12	Aminosol 5%	0.39												
		and	and	0.49	44,44	44	45	44	60	49	44				
		NH_4Cl	0.10												
	5/13	NH_4Cl†	0.10	0.08	44,44	62	64	48							
	5/14	Glutamic acid† (0.24%) and	0.08 and	0.61	42	42	44	51	46						
		Aspartic acid (0.12%) and NH_4Cl	0.08 and 0.45	3.05						71	64				
J. C.	1/14	Glycine 2%	0.0095	0.031	69,70	70	70	71	115						

*Except to J. C., all solutions were given at constant rate of infusion.
†Given in 5 per cent glucose and water.

delayed increases in blood ammonium concentrations and the prompt increases were proportional to the quantity of ammonium chloride added (Fig. 2 and Table III). In Patient P. D., a quantity of ammonium chloride sufficient to produce a prompt blood NH_4-N increase by itself (Fig. 3) was added to the protein hydrolysate. However, the resulting solution did not cause a prompt increase in this patient (Aminosol + NH_4Cl, Fig. 3). This last observation with slow infusions suggested that the ammonium analyzed in a protein hydrolysate could be neutralized by other substances contained in the same solution.



	NH_4N MG./ML.	TOTAL N MG./ML.
1.	0.454	5.8
2.	0.08	0.23
3.	0.08	0.13
4.	0.45	0.45
5.	0.400	6.1
6.	0.10	0.10
7.	0.0037	5.8

Fig. 3.—Venous blood ammonium in cirrhotic Patient P. D. during infusions of 5 per cent Aminosol, ammonium chloride, and different amino acids.

An ammonium neutralization or detoxification reaction probably would not occur in vitro but might occur in vivo where energy could be provided. The protein hydrolysate employed here contained both glutamic and aspartic acids which can combine with ammonium in vivo to form glutamine and asparagin, respectively.[10, 11] A solution was made up which contained both glutamic and aspartic acids in the concentrations analyzed in the protein hydrolysate.* To this solution was added ammonium chloride which pro-

*Analyses of Abbott Laboratories: Glutamic acid 0.24 per cent; aspartic acid 0.12 per cent.

vided more NH_4-N than found by analysis in the solution of Aminosol with added ammonium chloride given Patient P. D. (Fig. 3). The resulting solution (NH_4Cl + glutamic and aspartic acids) was given to the same patient at rates of 1.0 and 5.0 ml. per minute (Fig. 3 and Table III). No rise in blood NH_4-N concentrations was noted until 45 minutes after the slow rate infusion was started and a prompt second rise was observed 15 minutes after the infusion rate was increased fivefold. This finding provides indirect evidence that at slow infusion rates of the protein hydrolysate, Aminosol, either or both the glutamic and aspartic acids contained therein could prevent a prompt rise in this patient's blood NH_4-N concentration. At a faster rate, however, these decarboxylic amino acids were unable to prevent a prompt blood NH_4-N increase.

DISCUSSION

The above studies indicate that intravenous protein hydrolysates given rapidly can have an effect similar to orally administered protein in that either may bring on the syndrome of impending hepatic coma in susceptible patients with cirrhosis. In the case of the protein hydrolysates, this syndrome was presumably precipitated by ammonium contained therein and that produced in vivo by their administration. Investigations on the intravenous administration of animo acids and protein hydrolysates to dogs appeared recently which support this concept.[12, 13] Whether slow hydrolysate infusions delivering the same quantity of NH_4-N over a longer period would have produced impending coma was not investigated.

During constant rate infusions of the ammonium-containing or liberating substances employed, it was noted often that a decrease in blood NH_4-N concentrations occurred following a primary rise (Patient J. A., Fig. 1; Figs. 2, 3, and Table III). This secondary decrease was dependent neither on the height of the primary rise nor on the quantity of ammonium infused. These observations suggest the existence of an adjustable ammonium removal mechanism(s).

The delayed blood NH_4-N increases observed with slow infusions of both protein hydrolysate and individual amino acid solutions probably resulted from deamination of the infused amino acids. However, the report that urea infusions could cause rising blood NH_4-N concentrations in patients with cirrhosis[4] suggests that some of the substances infused in a protein hydrolysate could have affected certain body compounds or systems in such a way that either more ammonium was produced or, possibly, less detoxified.

That either or both the glutamic and aspartic acids in a protein hydrolysate solution could prevent a prompt blood ammonium increase when this solution was given slowly intravenously seems reasonable.[10, 11, 14, 15] In the present study, the quantities of these decarboxylic amino acids contained in the protein hydrolysate were insufficient to combine with all of the analyzed ammonium on a mole for mole basis. Therefore, it might appear that a prompt rise in blood NH_4-N did not occur at slow infusion rates of protein hydrolysate either because of an in vivo turnover of the infused glutamic

and aspartic acids (because the concentration of infused ammonium remaining after equimolar neutralization by these amino acids was insufficient to cause an increased blood NH_4-N concentration) or because, in addition to glutamic and aspartic acids, the protein hydrolysate contained other ammonium neutralizing or disposing compounds. The last mechanism suggested might well be operative in this study because the infused hydrolysate contained arginine (0.29 per cent), an animo acid which has been reported recently to aid in ammonium detoxification by increasing urea synthesis.[13, 16] Any or a combination of these postulated mechanisms might have been inadequate to prevent the prompt blood NH_4-N increases which occurred when the hydrolysates were infused at a sufficiently rapid rate.

If the above observations are confirmed in more patients, one might speculate that either the removal of ammonium from or the addition of ammonium-neutralizing or ammonium-detoxifying substances to protein hydrolysates might result in more satisfactory products for the parenteral administration of protein to patients with severe liver disease.

SUMMARY

1. During rapid rate infusions of 2 different protein hydrolysate solutions, 2 patients with hepatic cirrhosis developed a clinical syndrome indistinguishable from impending hepatic coma. This syndrome was associated with markedly increased venous blood ammonium concentrations.

2. Significant concentrations of ammonium nitrogen were found in 4 protein hydrolysate solutions studied.

3. One protein hydrolysate solution (5 per cent Aminosol) and solutions of L-glycine and DL-alanine produced delayed rises in blood ammonium concentrations when given slowly by the intravenous route. These delayed rises probably were caused by the deamination of the infused amino acids.

4. Slow rate infusions of 5 per cent Aminosol did not produce prompt rises in venous blood ammonium concentrations, although this hydrolysate was found to contain sufficient free ammonium to produce such an effect. The glutamic and/or the aspartic acids contained in this hydrolysate were sufficient to prevent the anticipated prompt increase in blood ammonium concentrations.

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THE EFFECTS OF INTRAGASTRIC ADMINISTRATION OF WHOLE BLOOD ON THE CONCENTRATION OF BLOOD AMMONIA IN PATIENTS WITH LIVER DISEASE

PHILIP C. YOUNG, M.D.,* CHARLES R. BURNSIDE, M.D., HARVEY C. KNOWLES, JR., M.D., AND LEON SCHIFF, M.D.
CINCINNATI, OHIO

INTRODUCTION

IN THE patient with liver disease, a number of conditions may precipitate hepatic coma. Included are the excessive use of alcohol,[1] the administration of ammonium compounds or diets high in protein content,[2-5] infections,[6] and abdominal paracenteses.[7] An association between gastroenteric hemorrhage and the development of hepatic coma has also been observed.[1, 7, 8] Since pathologic examination of the livers of patients dying under these circumstances has failed to reveal any characteristic lesions other than those of the primary liver disease, it has been suggested that this form of coma is related to the high blood ammonia concentration resulting from absorption of blood from the gastroenteric tract.[8] Accordingly, studies have been made on the concentrations of blood ammonia and other nitrogenous substances following the experimental introduction of blood into the stomach and have revealed a rise in the concentration of blood ammonia in the presence of liver disease.

METHODS

Observations were made on a control group and a group of patients with liver disease. The control group consisted of 8 normal male medical personnel of mean age 29 years (range 22 to 36). The liver profiles were normal in all the control subjects. In the group with liver disease there were 8 patients: 6 with cirrhosis, 1 with infectious hepatitis, and 1 with toxic hepatitis. Pertinent laboratory data are listed in Table I. The mean age of the patients with liver disease was 40 years (range 24 to 55). They were free of renal disease as gauged by urinalysis and the plasma concentrations of urea nitrogen and nonprotein nitrogen.

In each of the control subjects 500 ml. of blood was removed by venesection. In 4 subjects the blood was then immediately introduced into the stomach by Levine tube over a period of 30 minutes. In the remaining 4 subjects the blood was stored under refrigeration for 5 to 7 days before administration. In the group with liver disease venesection was not performed; instead the patients received 500 ml. of stored blood by Levine tube over a 30-minute period in a fashion similar to that of the control subjects. The amount of free ammonia found in the stored blood (average 340 gamma per cent) was considered insufficient to account for changes in the blood of the recipient. All subjects of both groups were in the fasting state at the start of the experiment. A light protein-free meal was provided 4 hours after the blood ingestion.

From the Gastric and the Metabolism Laboratories of the Department of Medicine, University of Cincinnati College of Medicine, and the Cincinnati General Hospital.

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*Trainee, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, Bethesda, Md.

TABLE I. LIVER FUNCTION AND RELATED TESTS IN THE PATIENTS WITH HEPATIC DISEASE

SUBJECT	AGE	THYMOL TURBIDITY (UNITS)	THYMOL FLOCCULATION	CEPHALIN FLOCCULATION	ZINC TURBIDITY (UNITS)	SERUM BILIRUBIN DIRECT/TOTAL (MG. PER CENT)	SERUM LIPIDS (MG. PER CENT)	ALKALINE PHOSPHATASE (BODANSKY UNITS)	SERUM PROTEINS (GM. PER CENT)	SERUM ALBUMIN (GM. PER CENT)	SERUM GLOBULIN (GM. PER CENT)	DIAGNOSIS
M. M.	46	13	4+	4+	33	0.3/0.7	630	4.8	7.45	2.0	5.45	Nutritional cirrhosis
M. A.	42	5	0	1+	10	1.5/2.9	540	5.3	6.50	2.7	3.80	Toxic hepatitis
Z. G.	24	31	4+	3+	43	0.6/1.2	915	8.7	7.25	2.3	4.95	Nutritional cirrhosis
W. P.	42	8	0	0	25	0.2/0.5	640	3.0	7.25	2.8	4.45	Nutritional cirrhosis
J. P.	27	11	4+	4+	26	5.0/9.0	575	2.8	7.25	3.5	3.75	Infectious hepatitis
J. W.	37	8	0	4+	6	3.0/5.5	1355	7.4	6.85	3.4	3.45	Nutritional cirrhosis
G. M.	55	22	4+	4+	28	0.5/1.5	855	4.3	6.00	2.05	3.95	Nutritional cirrhosis
V. T.	46	4	1+	4+	6	1.3/2.5	1115	4.0	6.9	2.3	4.60	Nutritional cirrhosis

Venous blood was drawn in heparinized syringes for determinations of blood ammonia, plasma urea nitrogen, and nonprotein nitrogen in the fasting state and at intervals of 1, 2, 3, 6, and 9 hours following ingestion of the blood.* The blood ammonia nitrogen content was determined in triplicate by a modification of the microdiffusion method of Conway.[11] Conway No. 2 dishes were prepared by placing 1 ml. of a saturated solution of potassium carbonate in the outer chamber and 1 ml. of a 1 per cent boric acid solution containing 1 drop of 1 per cent bromcresol green-methyl red indicator in the center wall. One milliliter of heparinized blood was introduced into the outer chamber within 30 to 45 seconds of blood letting. The dishes were sealed and diffusion was permitted to continue for 10 minutes at room temperature. Titration of the ammonium borate was then carried out with approximately 0.003 N hydrochloric acid. During each experiment standard curves were plotted using standard solutions containing 50, 100, 200, and 300 μg per cent ammonia nitrogen. At concentrations of 100 μg per cent or less the average deviation from the mean was ±4.8 per cent, and above 100 μg per cent, ±2.0 per cent. The heparinized blood was centrifuged and plasma separated for later determinations (in duplicate) of plasma urea nitrogen[12] and nonprotein nitrogen.[13]

RESULTS

In Tables II and III are given the concentrations of blood ammonia nitrogen, plasma urea nitrogen, and nonprotein nitrogen found in the control sub-

TABLE II. CONCENTRATIONS OF BLOOD AMMONIA NITROGEN, PLASMA UREA NITROGEN, AND PLASMA NONPROTEIN NITROGEN FOLLOWING INTRAGASTRIC BLOOD IN NORMAL SUBJECTS

SUBJECT	TIME (HOURS)					
	0	1	2	3	6	9
	Ammonia (μg Per Cent)					
H. B.	35	65		45	50	25
P. S.	33	16	15	62	62	43
C. B.	25	37	37	40	36	37
P. Y.	32	39	80	65	28	20
R. B.	15	15	30	60	13	20
R. W.	30	35	40	47	25	17
T. S.	20	33	55	45	27	15
D. R.	8	10	20	23	23	8
Mean	25	31	40	48	33	23
	Plasma Urea Nitrogen (Mg. Per Cent)					
H. B.	15	16		17	18	17
P. S.	19	18	17	17	20	22
C. B.	10	11	11	14	10	17
P. Y.	14	15	17	18	22	24
R. B.	16	16	18	21	25	24
R. W.	15	15	18	18	21	20
T. S.	10	13	13	15	15	14
D. R.	16	16	17	19	21	21
Mean	14	15	16	17	19	20
	Plasma Nonprotein Nitrogen (Mg. Per Cent)					
H. B.	19	36		38	40	32
P. S.	29	23	23	32	32	34
C. B.	26	34	38	34	34	34
P. Y.	32	41	47	44	47	44
R. B.	33	37	52	52	42	41
R. W.	34	39	42	40	40	34
T. S.	26	34	39	42	35	31
D. R.	32	39	50	50	43	41
Mean	29	35	42	42	39	36

*Arterial blood ammonia determinations were not carried out. Fortunately no difference exists in the ammonia content of venous and arterial blood in normals and the venous concentration is actually lower than the arterial in cases of cirrhosis of the liver.[9, 10] Hence the results of this study would not be invalidated by the omission of arterial blood determinations.

jects and those with liver disease. In Fig. 1, the mean values of the two groups are plotted.

In the normal subjects the mean blood ammonia nitrogen concentration rose slightly but significantly from a control value of 25 μg per cent to a maximum of 48 μg per cent ($P < 0.01$) in 3 hours, and returned to normal at 9 hours. The concentrations of plasma urea nitrogen slowly rose from a mean value of 14 mg. per cent at zero hour to 20 mg. per cent at 9 hours. The concentrations of plasma nonprotein nitrogen increased from a mean control level of 29 mg. per cent to a maximum of 42 mg. per cent within 2 hours. It remained elevated an additional hour and was decreasing at 6 hours.

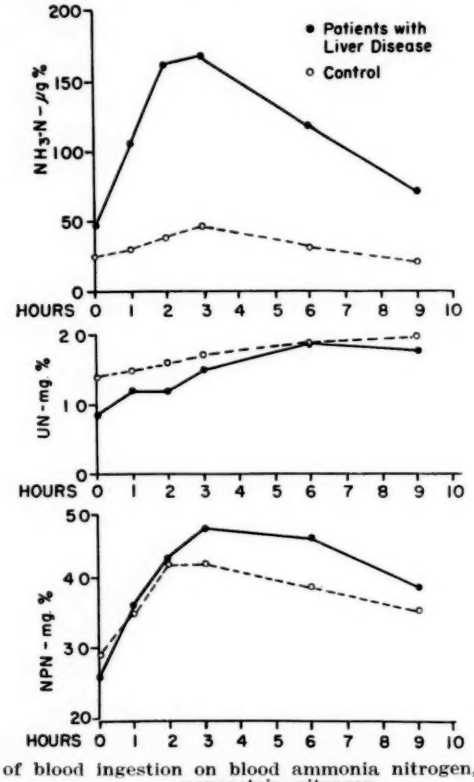

Fig. 1.—The effect of blood ingestion on blood ammonia nitrogen, plasma urea nitrogen, and nonprotein nitrogen.

In the patients with liver disease, the mean initial concentration of blood ammonia nitrogen of 47 μg per cent was significantly greater than that of 25 μg per cent obtained in the normals ($P < 0.01$). Following ingestion of blood, the mean concentration of blood ammonia nitrogen in the group of patients with liver disease rose to 167 μg per cent and remained significantly higher than that of the controls throughout the experiment. The mean concentration of plasma urea nitrogen increased from a control level of 9 mg. per cent to a maximum of 19 mg. per cent at the end of the 6-hour period of observation. The plasma nonprotein nitrogen concentration increased from a mean control value of 26 mg. per cent to 47 mg. per cent in 3 hours and then slowly dropped toward normal during the remaining 6 hours of study.

No significant differences between the curves of urea nitrogen and nonprotein nitrogen were found in the two groups ($P > 0.1$ for all patients). However, when the values of nonprotein nitrogen were considered as actual increases above a control level, the mean increases of 21 mg. per cent and 20 mg. per cent at 3 and 6 hours, respectively, in the group of patients with liver disease were significantly greater than those of 13 mg. per cent and 10 mg. per cent in the normals ($P < 0.01$, $P < 0.02$, respectively).

Three of the patients with decompensated cirrhosis became drowsy when ammonia levels were maximally elevated. A flapping tremor could not be demonstrated in any of the subjects.

TABLE III. CONCENTRATIONS OF BLOOD AMMONIA NITROGEN, PLASMA UREA NITROGEN, AND PLASMA NONPROTEIN NITROGEN FOLLOWING INTRAGASTRIC BLOOD IN PATIENTS WITH LIVER DISEASE

SUBJECT	TIME (HOURS)					
	C	1	2	3	6	9
			Ammonia (μg Per Cent)			
W. P.	50	83	115	150	104	72
M. M.	45	140	237	251	137	15
M. A.	50	115	170	200	114	70
Z. G.	15	95	200	115		50
J. P.	40	130		155	85	35
J. W.	80	95		172	40	46
G. M.	45	110	146	146	187	152
V. T.	47	70	95	145	160	133
Mean	47	105	161	167	118	72
			Plasma Urea Nitrogen (Mg. Per Cent)			
W. P.	4	15	7	10	11	6
M. M.	14	16	18	22	24	28
M. A.	4	5	8	9	11	13
Z. G.	8	9	10	9		14
J. P.	13	17		23	30	23
J. W.	5	9		10	12	12
G. M.	14	14	16	18	21	22
V. T.	11	12	13	15	21	22
Mean	9	12	12	15	19	18
			Plasma Nonprotein Nitrogen (Mg. Per Cent)			
W. P.	17	30	35	41	33	35
M. M.	39	59	65	66	57	55
M. A.	21	23	38	45	47	39
Z. G.	20	25	25	35		32
J. P.	31	46		49	57	38
J. W.	17	22		31	22	23
G. M.	32	42	50	57	52	44
V. T.	29	38	44	52	52	48
Mean	26	36	43	47	46	39

DISCUSSION

It is clear that the presence of blood in the gastroenteric tract of patients with liver disease causes a marked elevation of the blood ammonia concentration. A considerable portion of the blood ammonia content is derived from protein digestion in the bowel.[14] Amino acid oxidase and urease, protein splitting enzymes elaborated by coliform organisms, liberate ammonia within the intestine. The ammonia liberated is absorbed and transported to the liver through the portal system.[14, 15] Ammonia not utilized in the production of urea may escape

to the systemic circulation either by passage through the damaged liver or by way of collateral channels. The present experiments do not delineate the route mainly employed. Though collateral circulation may have partly accounted for the greater rise in nonprotein nitrogen in the patients with liver disease, inefficiency of deamination by a diseased liver may also have contributed to the increase. Moreover, in the patient with infectious hepatitis (J. P.), whose collateral circulation was presumably less marked than that of the patients with cirrhosis, the blood ammonia nitrogen concentration rose to a comparable level of 155 mg. per cent. Accordingly, it is highly probable that liver failure, per se, as well as collateral circulation, contributes to an increase in blood ammonia. In support of this is the observation that the increase of blood ammonia nitrogen concentration in patients with infectious hepatitis appears to be only slightly less than that found in cirrhosis.[15, 16]

CONCLUSION

The blood ammonia nitrogen concentration rises to high levels following absorption of blood from the gastroenteric tract in patients with liver disease.

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THE SERUM NEURAMINIC ACID DISTRIBUTION

I. Methodology

Abraham Saifer, M.S., and Shirley Gerstenfeld, B.S.
Brooklyn, N. Y.

BRAIN tissue is rich in many kinds of lipids, but only the cerebrosides and gangliosides contain carbohydrate radicals.[1] The cerebrosides yield mainly galactose on hydrolysis, whereas the gangliosides split off, in addition, a molecule of a complex amino acid-sugar called "neuraminic acid."[2] In amaurotic family idiocy (A.F.I.), or Tay-Sachs disease, there is an increase of the gangliosides up to 1.6 per cent of fresh tissue weight (normal value, 0.1 per cent), in some cases completely replacing cerebrosides or phospholipids of brain gray matter.[3] Klenk and his associates[4-7] were able to isolate methoxyneuraminic acid, as a cleavage product in crystalline form, both from normal and A.F.I. brain tissue and showed that it was responsible for many of the characteristic color reactions of the gangliosides. More recent work has shown that neuraminic acid is present in many biologic fluids and tissues usually in close association with a protein (glycoprotein) or lipid (glycolipid) material, e.g., blood serum,[8-11] milk,[12] urine,[13, 14] submaxillary mucin,[15, 16] red cell stroma lipids,[17, 18] colostrum glycoproteins,[19] lactating rat mammary gland tissue,[20] cerebrospinal fluid,[21] and in various animal glycoproteins[22, 23] including serum orosomucoids.[24, 25] According to a recent review article by Winzler,[26] it appears quite likely that all these investigators have been determining the same substance, or closely related ones, under a variety of names. Those most frequently referred to in the literature, in addition to "neuraminic acid,"[4] are "sialic acid"[16, 27] and the diphenylamine reacting substance of Dische[28] as applied to pathological sera.[29-31]

Winzler[26] describes neuraminic acid as "a component which has not yet been characterized chemically." However, recently Gottschalk[32, 33] has proposed structures for the methoxy derivative of neuraminic acid and for sialic acid which would account for all their relevant chemical reactions.

Three separate colorimetric reactions have been employed for the quantitative determination of neuraminic acid in biological material. These are: (a) the diphenylamine reaction[26, 30, 31]; (b) tryptophane–perchloric acid reaction[26, 34]; and (c) Bial's orcinol reaction.[5, 8] They all give characteristic reactions in the presence of a 2-deoxyhexose structure[35] which constitutes part of the neuraminic acid molecule.

From the Isaac Albert Research Institute, Jewish Chronic Disease Hospital, Brooklyn 3, N. Y.

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The present study deals with the development of a simplified diphenylamine procedure, for the determination of serum neuraminic acid, the normal values and reproducibility of results obtained with it.

EXPERIMENTAL

A. *Reagents and Apparatus.*—

1. Trichloracetic acid (T.C.A.) 10 per cent, 6 per cent, and 5 per cent solutions.

2. Sodium sulfite. 28 per cent solution.

3. Ether-span. Filter mixture of 1.0 ml. of Span 20* with 99 ml. of ether (reagent grade) through Whatman No. 1 filter paper and make up to the original volume with ether. Keep refrigerated when not in use.

4. Sodium chloride. 0.85 per cent solution.

5. Diphenylamine (D.P.A.) Reagent. 1.0 per cent. Recrystallize diphenylamine† twice from hot ethanol and dissolve 1.0 Gm. in mixture of 90 ml. of glacial acetic acid and 10 ml. of concentrated sulfuric acid.

6. Neuraminic acid standard. 0.20 mg. per milliliter. Obtain a purified orosomucoid protein sample from outdated blood bank plasma by the procedure of Weimer, Mehl, and Winzler.[36] This preparation has a reported neuraminic acid content of about 11.2 per cent and was checked against a reference sample.‡ Dissolve 21.7 mg. of the orosomucoid preparation in 10 ml. distilled water solution. This solution was stable over a 2-week period when frozen.

7. Evaporation caps to fit 15 × 150 mm. Pyrex tubes.§

B. *Procedure for Total Neuraminic Acid Content (T.N.A.) of Serum.*—Pipette, in duplicate, 0.10 ml. of each serum sample into 15 × 150 mm. test tubes. Add 5 per cent T.C.A. to each tube, with constant shaking, to a final volume of 5.0 ml. Pipette, in duplicate, 0.4 and 0.8 ml. aliquots of the orosomucoid standard solution, dilute with distilled water to 2.5 ml., then add 2.5 ml. of 10 per cent T.C.A. and mix by inversion. Cover each tube with an evaporation cap and heat in a boiling water bath for *exactly* 15 minutes. Cool tubes in cold tap water and centrifuge at 3,000 r.p.m. for 15 minutes. Pour off supernatant fluid into a small test tube but recentrifuge and decant into another tube if fluid is not perfectly clear.

Pipette 2.0 ml. aliquots of the clear fluids into 15 × 150 mm. test tubes. Add 4.0 ml. of the D.P.A. reagent to each tube and mix by tapping the bottom of the tube with a finger. Prepare a reagent blank by adding 2.0 ml. of 5 per cent T.C.A. to 4.0 ml. of D.P.A. reagent. Cover each tube with an evaporation cap and heat in a boiling water bath for exactly 30 minutes. Cool to room temperature in a cold water bath and read optical density values in a spectrophotometer at 530 mμ with the reagent blank set at zero.

Calculations: Neuraminic Acid (mg. per 100 ml. serum) $= \frac{\text{O.D. unk}}{\text{O.D. stnd}} \times \text{C}_{\text{stnd}} \times \frac{100}{.04}$

Since color reaction deviates from Beer's law, standard used in the calculation should be that which is closest to the unknown.

C. *Procedure for Neuraminic Acid Content of Globulin Fraction (G.N.A.) of Serum.*—Pipette, in duplicate, 6.0 ml. of 28 per cent sodium sulfite into 15 × 150 mm. test tubes and add 0.25 ml. of serum. Mix by inverting tubes once and then overlay with 3 to 4 ml. of ether-span. Mix 20 times by gentle inversion. (The tube is turned upside down and then back to its original position for one inversion.) Centrifuge for 10 minutes at 2,000 r.p.m. A button containing the total globulins is formed at the interface. By careful tilting, the button can be

*Atlas Powder Co., Wilmington, Del.

†Fisher Scientific Co., New York, N. Y.

‡This sample of known neuraminic acid content was generously provided for our use by Dr. R. J. Winzler of the University of Illinois College of Medicine.

§Obtainable from Metro Industries, Long Island City, N.Y.

disengaged from the side of the tube. A long thin capillary is inserted at an angle along the under side of the tube to the bottom and the supernatant fluid drawn off by means of gentle water suction. By means of a small polyethylene wash bottle, let a fine stream of water flow gently down the sides of the tube without disturbing the globulin button. Remove the wash water with the capillary pipette by gentle suction. Repeat this washing procedure twice more in order to remove as much of the albumin–sodium sulfite solution as is possible.

Pipette 1.0 ml. of 0.85 per cent sodium chloride into each tube and mix by tapping with finger in order to suspend the globulin fraction. Add 4.0 ml. of 6 per cent T.C.A., mix contents, cover with evaporation caps and place in boiling water bath for exactly 15 minutes. The remainder of this procedure is exactly as described in paragraph B for total serum neuraminic acid.

Calculations: Neuraminic Acid (mg. per 100 ml. serum) $= \frac{\text{O.D. unk}}{\text{O.D. stnd}} \times \text{C}_{\text{stnd}} \times \frac{100}{0.1}$

Use standard value closest to that of the unknown in calculating the results.

D. *Procedure for Total Protein, Albumin and Globulin Content of Serum.*—The method used for the albumin and globulin determination is that previously described by Saifer and Zymaris[37] which was checked against the moving-boundary electrophoretic method. Total proteins were determined with the biuret method.[38]

E. *Reproducibility of Serum Protein and Neuraminic Acid Values.*—A large pooled sample of human serum was divided into 9 portions by pipetting 5 ml. aliquots into test tubes, stoppering, and keeping them at –20° C. in a Deepfreeze unit. A tenth portion was run on the same day *without* freezing. The other samples were then thawed out at approximately 3-week intervals, over a 6-month period, and the serum protein and neuraminic acid values determined. The results obtained in these runs are shown in Table I, together with a statistical analysis of the data.

F. *Serum Protein and Neuraminic Acid Data of Normal Individuals.*—The serum protein and neuraminic acid values of a group of 37 normal individuals (26 adults and 11 children) were determined by means of the procedure described above. The data obtained in these runs are given in Table II. Since a preliminary analysis of the data showed no significant differences between the adult (Nos. 1 to 26) and children (Nos. 27 to 37) values, both groups were combined for purposes of statistical analysis.

DISCUSSION OF RESULTS

The diphenylamine method described here for serum neuraminic acid differs from those of Ayala,[30] Coburn,[31] and Winzler[26] in a number of essential features. In agreement with the latter, a purified glycoprotein (orosomucoid) of known neuraminic acid content is used as a standard instead of the color index values at 530 mμ as employed by Ayala[30] and Coburn.[31] Since known standards are included with each set of unknowns, there is increased reproducibility of results obtained in day-to-day runs as is shown by the data in Table I. The procedure has also been simplified from that of Winzler[26] by the elimination of the serum T.C.A.-filtrate blank, recommended by Coburn.[31] This blank, compared with the T.C.A.-diphenylamine blank, reads less than 0.01 optical density units. Therefore, its elimination saves serum and introduces no significant error in the method. The present technique also saves serum by using 0.1 ml. as compared to the 0.20 ml. recommended by Winzler.[26] While this has some importance where multiple biochemical studies on children are being performed,[39] of greater significance is the finding that considerably higher values of neuraminic acid were obtained when aliquots smaller than 0.20 ml. of 10

TABLE I. REPRODUCIBILITY OF PROTEIN AND NEURAMINIC ACID VALUES OF A FROZEN, POOLED SERUM SAMPLE

SAMPLE NUMBER	TOTAL PROTEIN (GM. PER CENT)	ALBUMIN (GM. PER CENT)	GLOBULIN (GM. PER CENT)	TOTAL NEURAMINIC ACID (MG. PER CENT)	GLOBULIN NEURAMINIC ACID (MG. PER CENT)	GLOBULIN NEURAMINIC ACID / GLOBULIN PROTEIN (PER CENT)	GLOBULIN NEURAMINIC ACID / TOTAL NEURAMINIC ACID (PER CENT)
1	7.00	3.62	3.38	92.9	59.9	1.77	64.5
2	7.00	3.60	3.40	88.3	57.8	1.70	65.5
3	6.92	3.70	3.22	96.5	54.5	1.69	56.5
4	7.06	3.73	3.33	90.5	55.1	1.66	60.9
5	7.10	3.60	3.50	91.1	56.4	1.61	61.9
6	7.16	3.59	3.57	95.5	55.1	1.55	57.7
7	7.04	3.48	3.56	96.2	59.7	1.67	62.0
8	6.92	3.46	3.46	93.6	54.9	1.59	58.6
9	6.86	3.66	3.20	92.6	53.4	1.67	57.6
10	7.02	3.41	3.61	97.5	56.9	1.58	58.4
Mean	7.01	3.59	3.42	93.5	56.4	1.65	60.4
± S.D.	0.08	0.10	0.13	2.8	2.0	0.063	2.9

normal sera were analyzed by the Winzler[26] method. This discrepancy of results is largely due to the fact that the change in optical density reading with increasing orosomucoid concentration does not follow Beer's law as is shown in Fig. 1. The concentration curve for this serum glycoprotein with the diphenylamine reaction is rather similar to that obtained by Anderson and Maclagan[14] for a glycoprotein isolated from normal male urine. Accurate calculation can, therefore, only be made by means of a calibration curve or by working at its lower end where it approximates Beer's law most closely.

The effect of variation of reagent concentrations, hydrolysis and heating times, acidity, etc., for the diphenylamine reaction has been adequately investigated both by Ayala et al.[30] and by Anderson and Maclagan.[14] Both groups of investigators obtained essentially the same spectral curves with a maximum at about 530 mμ although the former used human serum and the latter a glycoprotein derived from male urine. These results are also in agreement with those of Werner and Odin[40] who compared the spectral curves of serum to that of submaxillary mucin sialic acid with the diphenylamine reaction and found an absorption maximum for both at 530 mμ. In view of the agreement among three independent groups of investigators with respect to the factors which influence the diphenylamine reaction, it was felt that no further investigation of this matter was required in the present study.

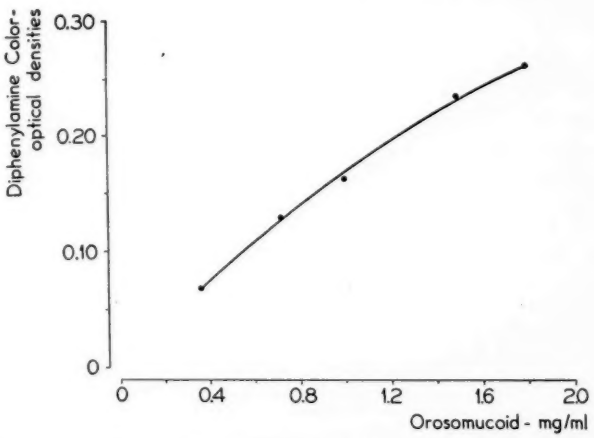

Fig. 1.—Calibration curve for serum neuraminic determinations with the diphenylamine reaction using orosomucoid solution as the standard.

Once the best conditions have been established for the reproducibility of the method for quantitative purposes, the question of the specificity of the reaction employed for neuraminic acid then arises. Possible substances present in biologic fluids which may interfere with the diphenylamine reaction were investigated by Anderson and Maclagan.[14] They concluded that only fructose, or fructose-containing compounds, gave strongly positive *blue* colors having an adsorption spectrum which might interfere with the purple diphenylamine color. However, we have confirmed the previously reported finding of Ayala et al.[30] that the trichloracetic acid filtrate from serum prior to hydrolysis gives no color with the diphenylamine reagent. Overend and Stacey[41] have reviewed

TABLE II. NORMAL SERUM PROTEIN AND NEURAMINIC ACID VALUES

SUBJECT NUMBER	TOTAL PROTEIN (GM. PER CENT)	ALBUMIN (GM. PER CENT)	GLOBULIN (GM. PER CENT)	TOTAL NEURAMINIC ACID (MG. PER CENT)	GLOBULIN NEURAMINIC ACID (MG. PER CENT)	GLOBULIN NEURAMINIC ACID / GLOBULIN PROTEIN (PER CENT)	GLOBULIN NEURAMINIC ACID / TOTAL NEURAMINIC ACID (PER CENT)
1	7.33	4.29	3.04	77.1	37.8	1.24	49.0
2	7.50	4.73	2.77	67.6	37.7	1.36	55.8
3	7.24	4.80	2.44	64.3	33.5	1.37	52.1
4	7.18	4.80	2.38	75.7	38.1	1.60	50.4
5	7.14	4.41	2.73	66.9	37.1	1.36	55.4
6	7.14	4.50	2.64	72.8	36.9	1.40	50.8
7	7.51	4.07	3.44	82.5	41.4	1.20	50.2
8	6.34	3.92	2.42	74.6	38.0	1.57	50.9
9	7.14	3.86	3.28	72.5	41.1	1.25	56.7
10	7.26	4.75	2.51	70.4	39.5	1.57	56.1
11	7.14	3.85	3.31	83.3	42.8	1.29	51.4
12	6.65	4.07	2.58	76.2	37.2	1.44	48 8
13	7.31	4.34	2.97	86.8	46.8	1.57	53.9
14	6.82	4.34	2.48	68.4	42.1	1.69	61.5
15	7.02	4.31	2.71	77.5	43.7	1.61	56.4
16	7.51	4.78	2.73	78.5	44.8	1.64	57.1
17	7.44	4.13	3.31	80.8	43.9	1.33	54.3
18	7.37	4.40	2.97	77.9	45.2	1.52	58.0
19	7.20	3.66	3.54	83.9	46.5	1.31	55.4
20	7.43	4.81	2.62	70.4	40.0	1.53	56.8
21	7.42	4.52	2.90	76.3	38.2	1.32	50.1
22	7.59	4.52	3.07	77.4	42.9	1.40	55.4
23	7.36	4.46	2.90	84.5	46.7	1.61	55.3
24	7.19	4.38	2.81	65.6	41.9	1.49	63.9
25	7.24	4.46	2.78	74.7	43.9	1.58	58.8
26	7.43	4.19	3.24	86.8	48.9	1.51	56.3
27	7.48	4.11	3.37	83.1	42.3	1.25	50.8
28	6.92	3.69	3.23	84.7	50.2	1.55	59.3
29	7.30	4.19	3.11	79.7	48.6	1.56	60.9
30	6.92	4.25	2.67	83.3	44.1	1.65	52.8
31	6.29	3.87	2.42	65.5	38.2	1.58	58.3
32	7.05	4.16	2.89	81.8	50.3	1.74	61.5
33	7.36	5.07	2.29	82.4	37.0	1.62	44.9
34	7.62	4.19	3.43	92.7	54.9	1.60	59.2
35	7.62	4.34	3.28	82.2	40.4	1.23	49.1
36	7.68	3.96	3.72	88.8	44.4	1.19	50.0
37	7.20	4.48	2.72	67.1	38.3	1.41	57.1
Mean	7.23	4.32	2.91	77.4	42.3	1.46	54.7
± S.D.	0.33	0.33	0.37	7.3	4.6	0.16	4.3

the chemistry of the 2-deoxysugars and discuss in detail the carbohydrate structures responsible for the color reactions with diphenylamine, tryptophane–perchloric acid, and Bial's orcinol reagent. Hess et al.[35] concluded that the purple color of the diphenylamine test is specific for 2-deoxyhexoses and hexals. The recent work of these investigators[35] substantiates the proposed structures of Gottschalk[33] for neuraminic acid as previously discussed. It can thus be concluded from the foregoing discussion, that neuraminic acid contains, as an integral part of the molecule, a 2-deoxyhexose or related structure. It is this grouping which is responsible for many of the colorimetric reactions used to identify its presence in the various glycoproteins found in many biologic substances including serum.[35, 41] The nomenclature with respect to proteins conjugated to carbohydrates is presently in a confused state. Some attempts to clarify the classification of such substances have been made by Winzler[26] and by Anderson and Maclagan.[14] It is the classification suggested by the former investigator which is employed in this paper.

As is seen in Table I, the serum protein and neuraminic acid values obtained with the procedure described in this paper show excellent reproducibility when the same sample is analyzed repeatedly. These results also indicate that a sample may be kept frozen for as long as 6 months, and that, if then thawed out and used immediately, no significant changes in the results occur. The statistical data at the bottom of the table permit one to calculate the expected confidence limits due to the experimental procedure itself, e.g., Mean ± t (at P = .05) × S.E. of mean are the 95 per cent confidence limits for the average. The average differences between duplicates is 3.3 per cent for total neuraminic acid, 5.4 per cent for the globulin neuraminic acid: total neuraminic acid ratio, and 4.2 per cent for the globulin neuraminic acid: globulin protein ratio.

The results obtained for serum protein and neuraminic acid levels in a group of 37 normal individuals are presented in Table II. This group comprised 26 adults of both sexes ranging in age between 22 to 50 years and 11 children from 1 to 6 years in age. The total serum neuraminic acid values of this table correspond to the color indices obtained with the diphenylamine reaction by Ayala et al.[30] and Coburn et al.[31] except for the use of orosomucoid as a standard. The values in this table, therefore, are expressed as milligrams of neuraminic acid per 100 ml. serum instead of in optical density readings. For the reasons previously discussed the mean value of 77.4 ± 1.2 (S.E.) obtained in this study is considerably higher than that reported by Winzler[26] of 60 ± 3.1 (S.E.) for 20 normal sera. It is also somewhat higher than the value of 66 ± 3.7 reported by this investigator when the tryptophane–perchloric acid reaction was used in place of the diphenylamine reaction to determine the neuraminic acid levels of presumably the same normal sera. Again these lower values are probably explainable on the basis of a deviation from Beer's law between the unknown and standard readings as well as to the decreased specificity of the tryptophane–perchloric acid method for serum neuraminic acid.

SUMMARY

1. The widespread presence of neuraminic acid and/or other related amino acid-sugars, e.g., sialic acid, hematoaminic acid, lactaminic acid, etc., in association with proteins or lipids (glycoproteins, mucoproteins, mucoids, glycolipids) indicates them to be substances of considerable physiologic importance.

2. A simplified method is presented for the quantitative determination of the neuraminic acid content of human serum and its globulin fraction, as obtained by salt fractionation, with the colorimetric diphenylamine reaction of Dische.[28]

3. The excellent reproducibility of the method for purposes of clinical studies was determined by analyses of 10 aliquots of a frozen, pooled serum sample over a 6-month time interval.

4. Serum values obtained with the procedure for 37 normal individuals (26 adults and 11 children) were as follows: total serum neuraminic acid, 77.4 ± 7.3 (S.D.) mg. per cent; globulin neuraminic acid, 42.3 ± 4.6 (S.D.) mg. per cent; globulin neuraminic acid: total neuraminic acid, 54.7 ± 4.3 (S.D.) per cent; globulin neuraminic acid: globulin protein, 1.46 ± 1.6 (S.D.) per cent. These normal serum results are somewhat higher than those obtained by Winzler and reasons for this discrepancy are discussed.

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THE SERUM NEURAMINIC ACID DISTRIBUTION

II. Clinical Studies With Special Reference to Amaurotic Family Idiocy (Tay-Sachs Disease)

Bruno W. Volk, M.D., Stanley M. Aronson, M.D., and Abraham Saifer, M.S.
Brooklyn, N. Y.

DURING the course of a study concerned with the clinical and morphologic evolution of amaurotic family idiocy (Tay-Sachs disease), parallel serial biochemical analyses of serum and cerebrospinal fluid were carried out.[1, 2] In most instances the afflicted children were institutionalized shortly after clinical inception of the disease and remained under observation until death, thus permitting protracted investigation. The serum protein electrophoretic data reported in this latter study[2] are summarized graphically in Fig. 1. Of particular significance is the increase in the alpha-2 globulins since many of the isolated serum glycoproteins have been found to migrate with an electrophoretic mobility characteristic of these proteins in barbital buffer at pH 8.6.[3-5] In addition, alpha-2 lipoproteins have been shown to be present in plasma by means of preparative zone electrophoresis.[6]

Klenk and his associates[7, 8] have demonstrated a new type of carbohydrate-containing lipid (gangliosides) in the cerebral gray matter derived from cases of amaurotic family idiocy (A.F.I). These lipids are characterized by the presence of a complex amino acid-sugar called "neuraminic acid"[9, 10] which is also found in serum in association with the glycoproteins.[11-13] A number of investigators have reported distributional changes in the various serum glycoproteins associated with different protein fractions in pathologic as compared to normal sera.[14, 15]

The present study was undertaken to investigate the relative distribution of neuraminic acid in the protein fractions of serum from patients with A.F.I., in comparison with normal serum, employing starch block electrophoresis. While this technique was successful in demonstrating a relative increase in the serum glycoproteins associated with the more slowly migrating electrophoretic protein fractions (Fig. 2) it is neither practical for routine clinical analyses nor for quantitative studies. It was for these reasons that the previously described salt fractionation procedure[16] was used to determine the neuraminic acid distribution within the albumin and globulin fractions of serum in A.F.I. as

From the Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, Brooklyn 3, N. Y.

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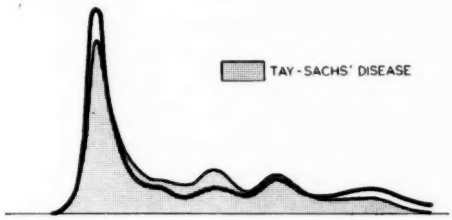
compared to groups of patients with other neurologic and nonneurologic conditions. The results obtained in this study show that A.F.I. can be distinguished by means of group statistics from other categories of diseases studied.

MATERIAL AND METHODS

Selection of Patients.—This study included 148 subjects who for purposes of group statistics were divided into six categories.



	Albumin gm%	Globulins gm%			
		alpha-1	alpha-2	beta	gamma
TAY-SACHS' DISEASE	3.70	0.50	0.98	0.96	0.65
NORMAL	4.07	0.48	0.66	0.96	1.01
P	<.01	.40	<.01	-	<.01

Fig. 1.—Serum protein electrophoretic pattern in amaurotic family idiocy (Tay-Sachs disease) as compared with that obtained for normal serum.

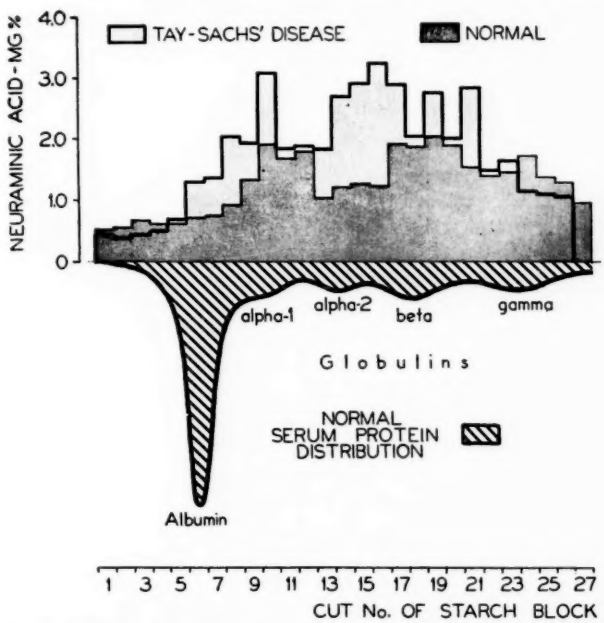


Fig. 2.—Distribution of serum neuraminic acid levels associated with various protein fractions as determined by starch block electrophoresis of a patient with amaurotic family idiocy as compared to a normal individual.

Group 1 (Twenty-five patients with debilitating diseases of nonneurological origin): This group included 9 patients with diabetes mellitus, 8 patients with chronic rheumatoid arthritis, 6 patients with generalized carcinomatosis of varying origin, 1 patient with muscular dystrophy, and 1 patient with hypertensive cardiovascular disease. All patients of this group were markedly debilitated.

TABLE I. GROUP STATISTICAL DATA OF SERUM PROTEINS AND NEURAMINIC ACID LEVELS IN 111 PATIENTS WITH VARIOUS DISEASES AS COMPARED WITH A GROUP OF 37 NORMAL SUBJECTS

DISEASE GROUPS		TOTAL PROTEIN (GM. PER CENT)	ALBUMIN (GM. PER CENT)	GLOBULIN (GM. PER CENT)	TOTAL NEURAMINIC ACID (MG. PER CENT)	GLOBULIN NEURAMINIC (MG. PER CENT)	GLOBULIN NEURAMINIC ACID / GLOBULIN PROTEIN (PER CENT)	GLOBULIN NEURAMINIC ACID / TOTAL NEURAMINIC ACID (PER CENT)
1. Debilitating nonneurologic diseases (25 patients)	*	6.84 ± .15	3.36 ± .18	3.48 ± .16	95.6 ± 3.7	54.0 ± 2.3	1.58 ± .043	56.5 ± 1.1
	†	6.42 – 7.26	2.87 – 3.85	3.03 – 3.93	85.4 – 106.	47.5 – 60.4	1.46 –1.70	53.3 – 59.7
2. Amaurotic family idiocy (8 patients—36 determinations)	*	6.96 ± .14	4.02 ± .14	2.94 ± .08	85.6 ± 2.0	55.1 ± .75	1.89 ± .027	64.7 ± 1.2
	†	6.48 – 7.44	3.52 – 4.52	2.66 – 3.22	78.7 – 92.5	52.5 – 57.7	1.80 – 1.98	60.6 – 68.9
3. Diffuse degenerative diseases of C.N.S. (45 patients)	*	7.24 ± .09	3.94 ± .06	3.30 ± .12	85.1 ± 2.1	48.3 ± 1.3	1.51 ± .048	56.9 ± .82
	†	7.00 – 7.48	3.78 – 4.10	2.99 ± 3.61	79.6 – 90.6	45.0 – 51.6	1.39 – 1.63	54.8 – 59.0
4. Hemiplegia (10 patients)	*	7.07 ± .20	3.81 ± .14	3.26 ± .14	93.0 ± 4.1	51.2 ± 2.8	1.59 ± 0.11	55.0 ± 1.6
	†	6.43 – 7.71	3.37 – 4.25	2.79 – 3.73	79.8 – 106.	42.1 – 60.3	1.24 – 1.94	49.7 – 60.3
5. Postinfectious poliomyelitis (23 patients)	*	7.24 ± .08	4.42 ± .13	2.82 ± .08	81.2 ± 1.9	43.3 ± 1.1	1.56 ± .050	53.6 ± 1.2
	†	7.02 – 7.46	4.06 – 4.78	2.59 – 3.05	75.9 – 86.5	40.1 – 46.5	1.42 – 1.70	50.2 – 57.0
6. Normal subjects (37 individuals)	*	7.23 ± .05	4.32 ± .06	2.91 ± .06	77.4 ± 1.2	42.3 ± .76	1.46 ± .026	54.7 ± .71
	†	7.09 – 7.37	4.18 – 4.46	2.75 – 3.07	74.3 – 80.5	40.3 – 44.3	1.39 – 1.53	52.9 – 56.5

*Mean ± Standard Error (S.E.).

†Estimated range of population (probability at 0.01 level) = Mean ± t (S.E.).

Group 2 (Eight patients with amaurotic family idiocy): The children comprising this group were all of Jewish ancestry and their clinical course was characterized by arrested development, macular degeneration and amaurosis, progressive paresis, convulsive seizures, and inanition. Of the 4 patients who died at ages varying from 18 to 36 months, the diagnosis was confirmed at autopsy in 3 of the children. The ages of the surviving children varied from 18 to 29 months at the time of termination of this study. Serial determinations of serum proteins and neuraminic acid levels were carried out at intervals of 6 to 8 weeks. Thus, from 1 to 9 sera were examined for each child with a total number of 36 specimens subjected to the various procedures. The values obtained for each patient in this group were averaged for purposes of group statistics.

Group 3 (Forty-five patients with degenerative diseases of the central nervous system): This group included 10 cases of multiple sclerosis, 2 patients with amyotrophic lateral sclerosis, 3 patients with degenerative cerebellar ataxia, 4 patients with spina bifida, 6 patients with dystonia musculorum deformans, etc.

Group 4 (Ten patients with hemiplegia): This group included cases with clinically evident hemiplegia of cerebrovascular etiology.

Group 5 (Twenty-three patients with postinfectious poliomyelitis): This category comprised patients with residua of paralytic poliomyelitis whose convalescent period varied from 3 months to 22 years prior to this study.

Group 6 (Thirty-seven normal subjects): This group, serving as a control, consisted of 26 adults (21 to 50 years old) and 11 children (18 months to 16 years old). All individuals included in this study were in good health.

Biochemical Studies.—

Serum proteins and neuraminic acid values: These were determined by the procedures described in Part I of this study.[16]

Starch block electrophoresis: The technique employed in these studies for starch block electrophoresis was essentially the same as that described in a recent review article by Kunkel.[17] The apparatus used was constructed so as to permit the use of silver-silver chloride electrodes and to allow runs to proceed for 48 hours or longer without contamination of the starch block by electrode by-products. The potato starch (Matheson) was premixed with, and the electrophoretic runs were performed in, barbital buffer, pH 8.6, 0.05 ionic strength. The design of the cell permitted 3 1.0 ml. serum samples to be run simultaneously, e.g., a normal control together with 2 pathologic sera. After the albumin front, which is visible under ultraviolet light, had moved a distance of about 20 to 25 cm., the current was shut off and the block cut up into a series of 30 to 35 one cm. sections. After elution of each starch cut with 2.0 ml. of distilled water and centrifugation, its protein content was determined on a 0.2 ml. aliquot with the colorimetric procedure of Lowry.[18] The neuraminic acid content of each cut was established on 0.5 ml. aliquots of the eluted serum proteins, after precipitation with an equal volume of 10 per cent trichloracetic acid, with the same technique as previously described for serum.[16] In this manner starch block electrophoresis was carried out on the individual sera of 6 normal subjects and of 6 patients with A.F.I. A typical set of results as obtained for one normal and one A.F.I. serum is shown in Fig. 2.

Statistical Methods.—The statistical analysis was carried out for the series of normal controls as well as for the 5 groups of patients by the techniques of group statistics.[19] The significant data obtained are summarized in Table I and are graphically illustrated in Figs. 3 to 6.

RESULTS

Significant changes in the serum proteins (inverted A/G ratio), as determined by salt fractionation methods,[20] are confined solely to the clinical cases comprising Group 1.[21]

The total serum neuraminic acid values are significantly elevated in Group 1 (nonneurologic debilitating diseases) and in Group 4 (hemiplegias). A lesser increase is observed in Group 2 (A.F.I.) and Group 3 (diffuse degenerative diseases of the central nervous system). The general nature of the increase in the total serum neuraminic acid values for most diseases, nonneurologic as well as neurologic, is illustrated in Fig. 3.

The serum globulin neuraminic acid results (Fig. 4) show a considerable elevation above normal in all the disease categories studied except for Group 5 (postinfectious poliomyelitis). The highest mean value of this component is now observed in the group of patients with A.F.I. When, on the other hand, the total serum neuraminic acid results are considered (Fig. 3), the highest mean value is shown by the patients of Group 1 with debilitating diseases of nonneurologic origin.

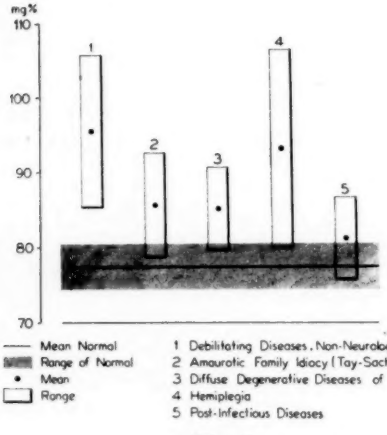


Fig. 3.

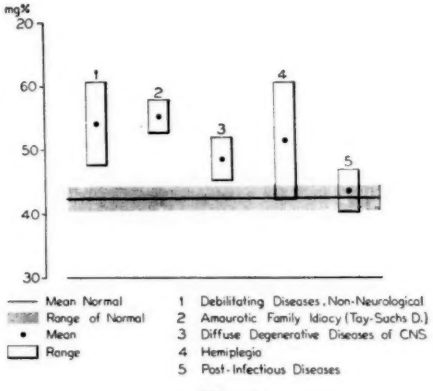


Fig. 4.

Fig. 3.—Total serum neuraminic acid values of patients in various disease groups as compared with a group of normal controls.

Fig. 4.—Serum globulin neuraminic acid values of patients in various disease groups as compared with a group of normal controls.

When the ratios of globulin neuraminic acid/serum globulin protein (G.N./G.P.) or globulin neuraminic acid/total serum neuraminic acid (G.N./T.N.) are calculated for each of the 6 groups (Figs. 5 and 6), the A.F.I. series (Group 2) becomes more clearly defined from those of the other 4 disease categories. When the G.N./G.P. is employed (Fig. 5), there is still some overlap between the A.F.I. and the hemiplegic group. However, the use of the G.N./T.N. ratio, as is shown in Fig. 6, serves to separate the A.F.I. patients from all the other disease categories studied.

The distribution of neuraminic acid among the various serum protein fractions in both normal and A.F.I. sera as determined by starch block electrophoresis is shown in Fig. 2. In normal subjects as well as in patients with A.F.I., neuraminic acid is distributed throughout the entire electrophoretic serum spectrum. An increase is observed in the A.F.I. serum neuraminic acid values in practically all the protein fractions when compared to normal serum. Of

greater significance, however, is the considerable shift in the distribution of neuraminic acid among the various protein fractions in the direction of those which migrate more slowly, i.e., from the alpha-1 into the alpha-2 and beta regions. These experiments with starch block electrophoresis substantiate the findings obtained with the salt fractionation technique discussed above. As previously mentioned, similar patterns to those shown in Fig. 2 were obtained with 6 normal and 6 A.F.I. sera studied with this procedure.

DISCUSSION

Neuraminic acid, a complex amino acid-sugar, is a constituent of many glycolipids and glycoproteins. Its widespread occurrence in many tissues and

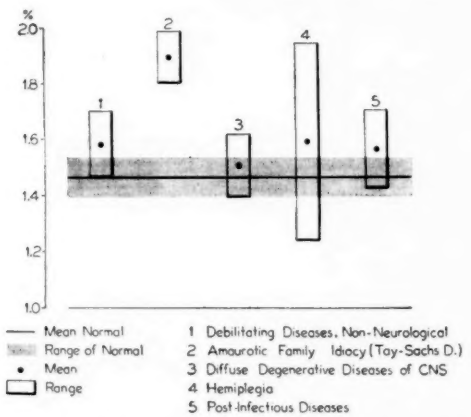


Fig. 5.—Ratio values of serum globulin neuraminic acid/globulin protein of patients in various disease groups as compared with a group of normal controls.

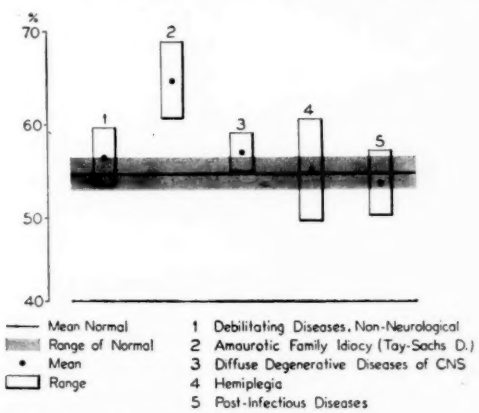


Fig. 6.—Ratio values of serum globulin neuraminic acid/total serum neuraminic acid of patients in various disease groups as compared with a group of normal controls.

biologic fluids of both man and animals indicates that it plays an important, if as yet undetermined, physiologic role. In a comprehensive review article, Chatagnon and Chatagnon[22] discuss the isolation of this substance by various groups of workers in different biologic materials including serum glycoproteins.

In general, isolated and purified glycoproteins from serum, urine, or other biologic materials have been shown to migrate electrophoretically with the mobility of serum alpha-1 or alpha-2 globulins. However, the work of Greenspan[15] has shown that glycoproteins are present in all the serum protein fractions, and that in various diseases these substances are not only increased, but that there is a shift in their relative concentration from one protein fraction to another. The presence of some glycoproteins, as measured by their neuraminic acid content, associated with serum protein fractions other than alpha globulins has been confirmed by the paper electrophoretic studies of Uzman and Rosen.[23] These authors found about 85 per cent of the neuraminic acid to be associated with the alpha or beta globulins and provide evidence that it is either bound to alpha-2 globulins or is present as a constituent of the polysaccharide or glycolipid moieties of the alpha-2 globulins. However, Chatagnon and Chatagnon[24] demonstrated by means of paper electrophoresis that neuraminic acid in both serum and urine was bound mainly to the alpha and gamma globulin fractions.

In our studies using starch block electrophoresis, neuraminic acid was bound to all the protein fractions, including albumin, with the largest proportion associated with the alpha and beta globulins. The selective increase in the neuraminic acid values of the alpha and beta globulins in A.F.I., as shown in Fig. 2, is in agreement with the findings of other investigators who observed a shift in the serum glycoprotein in pathologic sera employing different techniques.[15] The neuraminic acid results found by salt fractionation techniques are more akin to those obtained with starch block electrophoresis than with paper electrophoresis. This may be due to the fact that the adsorption of alpha and beta globulins as the main lipid-carrying fractions is stronger on paper than on starch.[17] The problem of the uniqueness of the protein-neuraminic acid starch block electrophoretic pattern for A.F.I. (Fig. 2) as compared to other neurologic diseases is presently under investigation. Recent work[26, 27] indicates the presence of more than one kind of gangliosides in brain gray matter and a different distribution of these gangliosides in A.F.I.

The problem of the specificity of the diphenylamine reaction for neuraminic acid has been discussed in some detail in Part I of these papers.[16] Work by other investigators has demonstrated conclusively that isolated crystalline neuraminic, or sialic acid, from either serum or various tissues, gives an identical absorption spectrum to that obtained when the diphenylamine reaction is applied to whole serum.[11-14, 28] The possibility cannot be denied that the diphenylamine reagent may react with unknown substances other than neuraminic acid in biologic fluids. However, recent work, as discussed in Part I,[16] has tended to confirm the high order of specificity of this reaction for neuraminic acid.

The diphenylamine reaction as applied to whole serum has been found significantly increased in a wide variety of pathologic conditions including malignant neoplasms, tuberculosis, rheumatic fever, rheumatoid arthritis, and also other infectious and inflammatory diseases.[25]

The nonspecific character of the increased total serum neuraminic acid values has been confirmed by our studies applied to a variety of neurologic

and nonneurologic diseases (Fig. 3). In view of the reported finding that neuraminic acid is markedly increased in the cerebral gray matter of patients with A.F.I., it is of interest that no singular increase in *total* serum neuraminic acid is found in this disease. On the contrary the highest levels were observed in the group of nonneurologic, debilitating diseases (malignant neoplasms, rheumatic fever, rheumatoid arthritis, etc.) and in patients with hemiplegia. It may, therefore, be suggested that the elevated total serum neuraminic acid in A.F.I. is causally unrelated to the storage of similar reacting substances in the distended neurons of this disease. A number of different hypotheses have been advanced to explain the increase in the serum glycoproteins found in various diseases.[14] Our findings in A.F.I. seem to be in general accord with the suggestion of Seibert et al.,[28] that elevations of this substance reflect processes of tissue destruction which parallel the increase in alpha-2 globulin levels as determined electrophoretically (Fig. 1). However, the increased G.N./T.N. ratio in this disease (Fig. 6), as compared to other pathologic conditions, indicates both a selective increase in the neuraminic acid-containing glycoproteins, or glycolipids, associated with the alpha-2 and beta globulin fractions (Fig. 2), as well as the possibility that these represent new substances resulting from the disease process.[26, 27] Further elucidation of this point must await additional experimental work on the tissues of patients with A.F.I.

The G.N./T.N. ratio, as elaborated in this study, seems to offer a useful diagnostic adjunct in confirming the diagnosis of A.F.I., particularly in early cases of this disease. While the statistical data shown here (Table I) apply to the group rather than to individual cases, two or more abnormal findings in the same patient appear highly suggestive of this disease. Similar studies applied to parents of children afflicted with A.F.I. are presently in progress in an attempt to explore further the potentially hereditary aspects of this disease.

SUMMARY

1. The quantitative distribution of serum neuraminic acid between the albumin and globulin protein fractions, as separated by salt fractionation, was investigated for 37 normal subjects (26 adults and 11 children) and for 111 patients with neurologic and nonneurologic diseases using a modified diphenylamine colorimetric reaction.

2. Because of the previously reported increase of neuraminic acid-containing lipids (gangliosides) in the cerebral gray matter from cases of amaurotic family idiocy (A.F.I.), serum determinations on the 8 patients in this group were carried out at 6- to 8-week intervals for a total of 36 separate analyses.

3. For purposes of group statistical analysis, the 111 patients were divided into 5 disease categories and compared against the normal control group. The total serum neuraminic acid was found to be elevated in all the groups studied, with the highest mean values present in the nonneurologic, debilitating diseases, e.g., rheumatic fever, rheumatoid arthritis, malignant neoplasms, etc.

4. The best statistical indicator for differentiating A.F.I. from all other disease groups was the serum globulin neuraminic acid/total serum neuraminic

acid ratio. That the increase in this ratio represents a relative shift of serum glycoproteins from the albumin to the globulin protein fractions was verified by means of starch block electrophoresis of both normal and A.F.I. sera.

5. The possible relationship between the markedly increased ganglioside content of the neurons of brain gray matter in A.F.I. and of the relative increase in serum glycoproteins (as determined by neuraminic acid content) associated with the alpha-2 and beta globulin fractions is discussed.

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LEUKOCYTE MOBILIZATION IN NORMAL, SPLENECTOMIZED, AND LEUKEMIC RATS AFTER REPLACEMENT TRANSFUSION

J. W. Hollingsworth, M.D.,* J. Alfred Berend, B.S.,** Daniel R. Silbert, B.S.,*** and Stuart C. Finch, M.D.****
New Haven, Conn.

THE rapid mobilization of leukocytes into the circulation after depletion by hemorrhage or by localization in areas of inflammation has been frequently noted. The extreme rapidity with which leukocyte depletion is corrected points to a redistribution of preformed mature leukocytes from storage depots, rather than new production, as the mechanism by which the blood leukocyte count is restored. The bone marrow maturation time for granulocytes probably is in the range of 3 to 4 days.[1, 2] Osgood[3] has estimated that man has more than forty times as many mature granulocytes in storage areas as in the circulating blood, and probably even more noncirculating lymphocytes. The sites of storage from which these cells reach the circulation is not known. Recent studies by Craddock and his associates[2] of dogs made leukopenic by autogenous "leukopheresis" have suggested that granulocytes entering the blood after acute leukocyte depletion come from mature cells in storage within the bone marrow. The role of storage depots within sluggish vascular channels or in tissue spaces has not been estimated.

We have investigated the response of normal, splenectomized, and leukemic rats to leukocyte removal by means of replacement transfusion with artificially prepared leukopenic blood. This method seemed ideally suited to our study because there are no erythrocyte blood group incompatibilities in laboratory rats and these animals are singularly unresponsive to stress factors and pyrogens that might affect the normal response. Since the normal rat has a predominance of lymphocytes, leukocyte replacement would yield more information about the available reserves of this type of blood cell. In these studies the pattern of response to leukocyte depletion of the normal rat was compared with that of splenectomized and leukemic animals.

From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut.

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*Research Assistant in Medicine.

**Senior Medical Student.

***Senior Medical Student.

****Assistant Professor of Medicine.

MATERIALS AND METHODS

Sprague-Dawley strain male rats weighing 250 to 300 grams were used in most experiments. The leukemic rats were of Sherman strain in which Shay granulocytic chloroleukemia had been induced by intraperitoneal injection of leukemic cell suspensions in newborn rats. The method of therapy that allowed remission of the leukemia until these animals reached the 200 to 300 gram size has been previously described.[4]

Replacement transfusion was performed by means of polyethylene cannulae (size PE 50) in the femoral artery and vein. Normal donor rats were exsanguinated from the heart, and the heparinized blood spun in a small angle centrifuge. The plasma was aspirated and saved, the buffy coat carefully aspirated and discarded and the plasma recombined with the packed red cells. The leukocyte count on the reconstituted donor blood almost always was less than 1,000 per cubic millimeter. This leukopenic blood was injected through the femoral vein cannula at the same rate as arterial blood flowed spontaneously from the cannula in the femoral artery. By this technique blood replacement occurred at the rate of 5 to 20 ml. per minute. Total and differential leukocyte counts from the femoral artery cannula were taken at desired intervals during and after blood replacement. Two types of experiments were designed to study the responses of the animals: (1) continuous rather massive (50 ml.) replacement and (2) intermittent smaller (20 ml.) replacements with 30-minute recovery periods. A total of 22 animals were studied by the single massive replacement procedure (6 normals, 5 acutely splenectomized, 2 chronically splenectomized, 5 sham operated splenectomy controls, and 4 leukemic). Fourteen animals were also studied by the technique of intermittent replacement transfusions (4 normals, 2 acutely splenectomized, 2 chronically splenectomized, and 6 leukemic). Since the results were quite similar both in individual experiments and in the two types of experimental procedures, the mean leukocyte values have been taken for analysis.

The efficacy of blood replacement was studied by means of radioactive sodium chromate labeling of the red cells used for replacement. Approximately 3 μc of $Na_2Cr^{51}O_4$ (Abbott) was incubated with 50 ml. of rat blood at room temperature for 30 minutes. The plasma was removed by centrifugation, the cells washed twice with 0.85 per cent sodium chloride, and the packed washed cells resuspended in normal rat plasma. Replacement transfusion was performed with the tagged donor blood and 0.1 ml. samples of blood from the arterial cannula of the recipient rat were obtained at intervals during replacement transfusion. The samples were counted in a well-type scintillation counter and compared with the activity of the original labeled donor blood.

RESULTS

A. *Single (50 Ml.) Replacement Transfusion.*—In 2 rats weighing 300 grams, the rates of replacement as determined by the Cr^{51}-tagged donor red cells were nearly identical (Fig. 1). The semilogarythmic decrease of the autogenous cell population as blood replacement continued was as expected. The decrease in autogenous red cells during replacement transfusion is compared with the mean decrease in leukocyte count of the groups of experimental animals during a single 50 ml. replacement (Fig. 1). During the acute phase of leukocyte replacement all animals exhibited some mobilization of storage leukocytes, as evidenced by the fact that the leukocyte count decreased less than would be expected in an adynamic system represented by the disappearance of autogenous erythrocytes. It is evident from the graph that the leukemic and chronic splenectomized animals exhibited a greater percentage fall

than did the other groups. This effect, however, appeared to be directly related to the initial high white cell level of these animals. In the group of normal animals, some of the rats had initial leukocyte counts of 4,000 to 6,000 per cubic millimeter and replacement transfusion caused minimal percentage drop in counts of these animals.

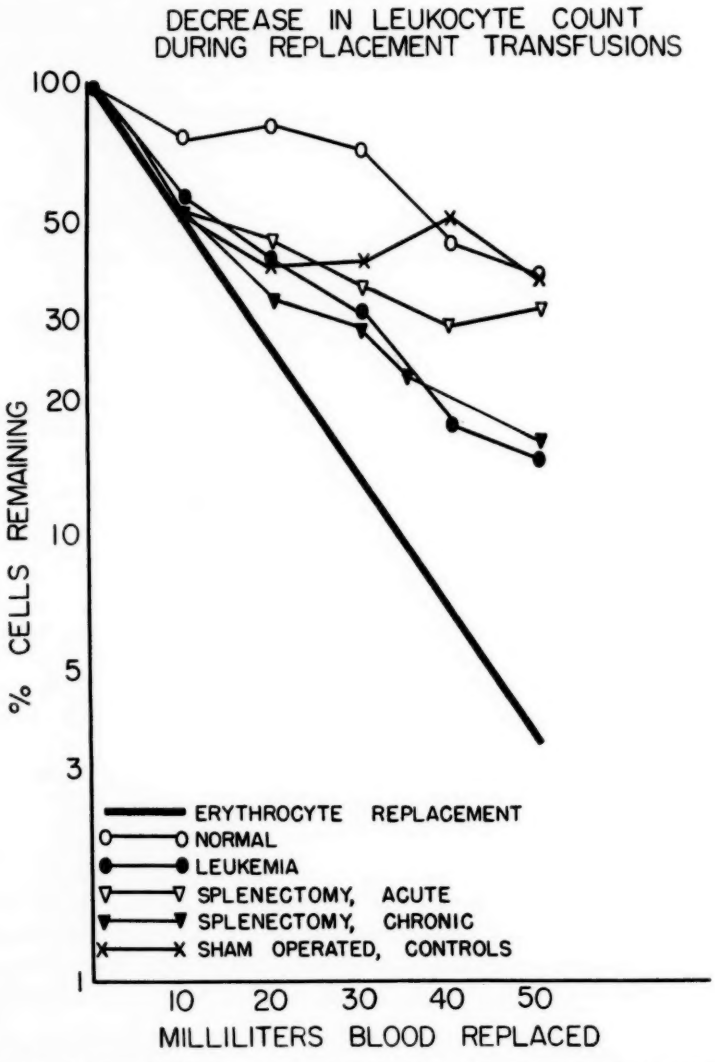

Fig. 1.—Leukocyte changes with massive (50 ml.) replacement transfusions. Leukocyte replacement with leukopenic blood was carried out at rates of 4 to 20 ml. per minute in 5 groups of animals. The mean percentage decrease in leukocyte count for each group during the procedure was compared with the decrease in an adynamic system as typified by the line of erythrocyte replacement.

In Fig. 2, the actual mean leukocyte counts of the 5 groups of animals are plotted, both during the procedure and for the succeeding 90 minutes. Although in these experiments the total quantity of leukocytes removed equaled approximately twice the total circulating leukocyte volume, normal animals

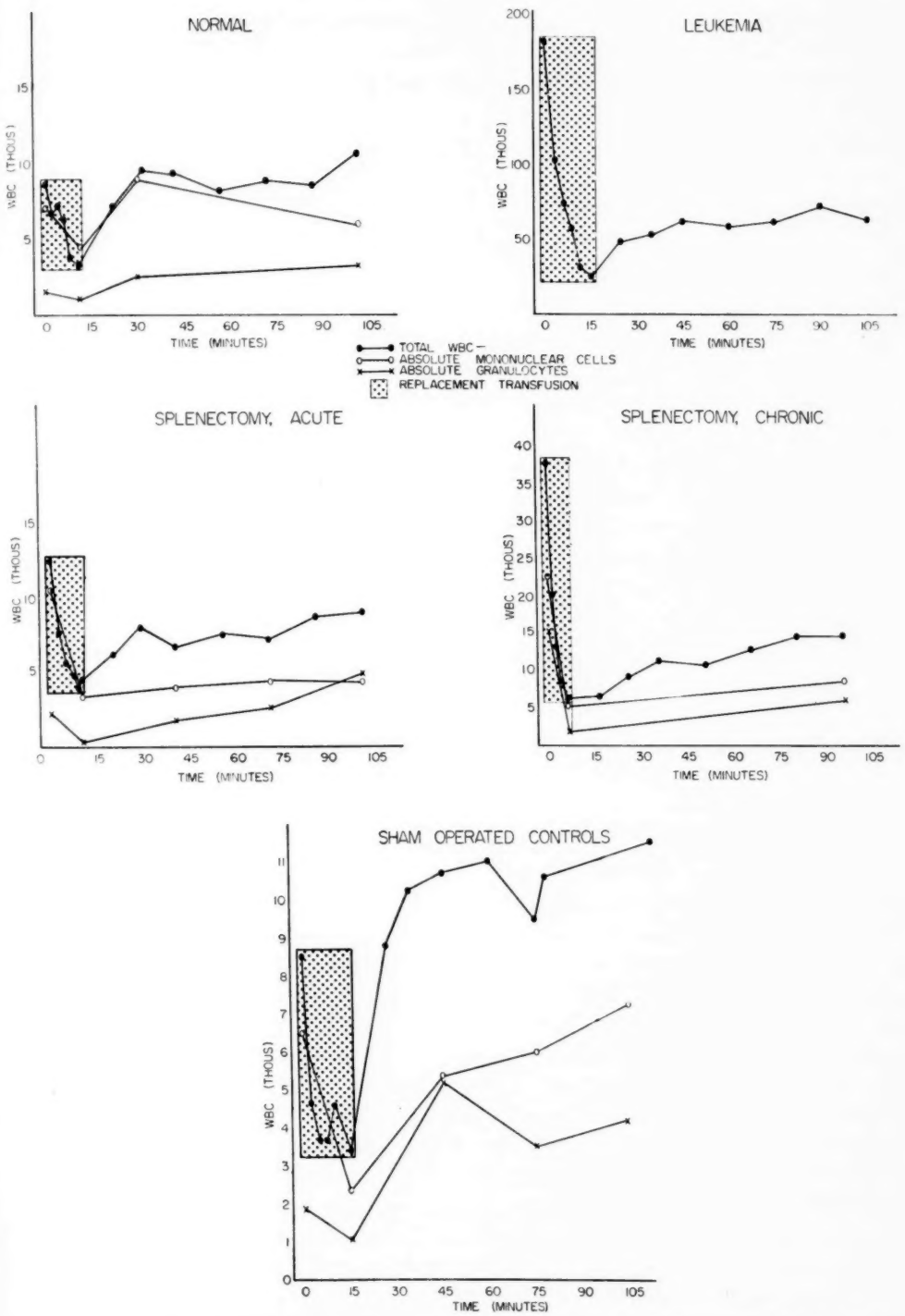

Fig. 2.—The mean total and differential leukocyte counts of 5 groups of rats during and after single leukocyte replacement transfusions are plotted above. Each stippled column represents the period during which a 50 ml. replacement transfusion was carried out.

regained the pretreatment leukocyte level within 20 minutes. Although differential leukocyte counts were not obtained in all animals at the 30-minute period after replacement, it was evident that this rapid replenishment of blood leukocytes in the normal rat was accomplished by both granulocytes and lymphocytes, so that the absolute levels of the 2 cell types had returned to the base-line level. Even at the end of the observation period there was little change in the leukocyte pattern.

In the animals that were acutely splenectomized (splenectomy within 10 minutes before the start of the replacement transfusion) the results were similar to those of the normal animals. However, the recovery of the leukocyte count was more delayed and the granulocyte level was increased at the end of the observation period. The sham-operated control group for the acute splenectomy series showed prompt return of the white cell count to above base-line levels.

The chronic splenectomized group (splenectomy 3 to 5 days before study) had a marked leukocytosis at the beginning of the study, consisting of absolute increases in both granulocytes and lymphocytes. In these animals replacement transfusion effected a marked decrease in leukocyte level, with return of the count only to normal levels during the observation period. The cells returning to the circulation consisted of both granulocytes and lymphocytes in a proportion approximating their initial ratios.

The leukocyte count of the leukemic rats also fell precipitously during replacement transfusion. There was an immediate return of cells into the circulation (22,000/c.mm. within 10 minutes), but later the increase was more gradual. The original leukocyte level was much higher than that obtained at the end of the 90-minute observation time following leukocyte removal.

B. *Intermittent (20 Ml.) Replacement Transfusions.*—The same experimental groups of animals (except for the sham-operated controls) were studied during 3 20 ml. replacement transfusions 30 minutes apart. This volume of blood represents about 80 per cent blood replacement. The mean leukocyte responses are plotted in Fig. 3. The replacement transfusions (depicted by the dotted areas) caused a prompt fall in leukocyte count of the normal animals each time, but during the 30-minute recovery period the counts returned to near base-line levels. During recovery, both granulocytes and lymphocytes were returned to the circulation in normal numbers. The mean counts in the last point on the chart are derived from only 3 of the 4 animals in the group, but it seems evident that only moderate granulocytosis occurred at the end of the experiment, 90 minutes after the last replacement transfusion.

The acutely splenectomized animals again exhibited less rapid recovery of the arterial leukocyte levels after replacement transfusion. Each replacement transfusion, therefore, depressed the white cell count progressively. The experiments failed to demonstrate a predominance of either lymphoid or granulocytic elements replenishing the circulation.

The animals that had been splenectomized for 3 to 5 days recovered only to a minimal extent after the replacement transfusions and their initial leukocytosis was decreased in stepwise fashion by the 3 replacement transfusions. As in the previous studies, although postsplenectomy granulocytosis was evident in the initial counts, both cell types participated in the leukocyte recovery periods.

Of the 6 leukemic animals studied, 4 had leukocyte counts below 100,000 per cubic millimeter, and 2 had very high leukocyte counts. The mean values of all 6 animals, as plotted in Fig. 3, reveal a sharp decrease in count with each replacement and poor recovery between procedures. It is of interest that the 2 leukemic animals with the very high leukocyte counts exhibited greater percentage fall with replacement and poorer percentage recovery than

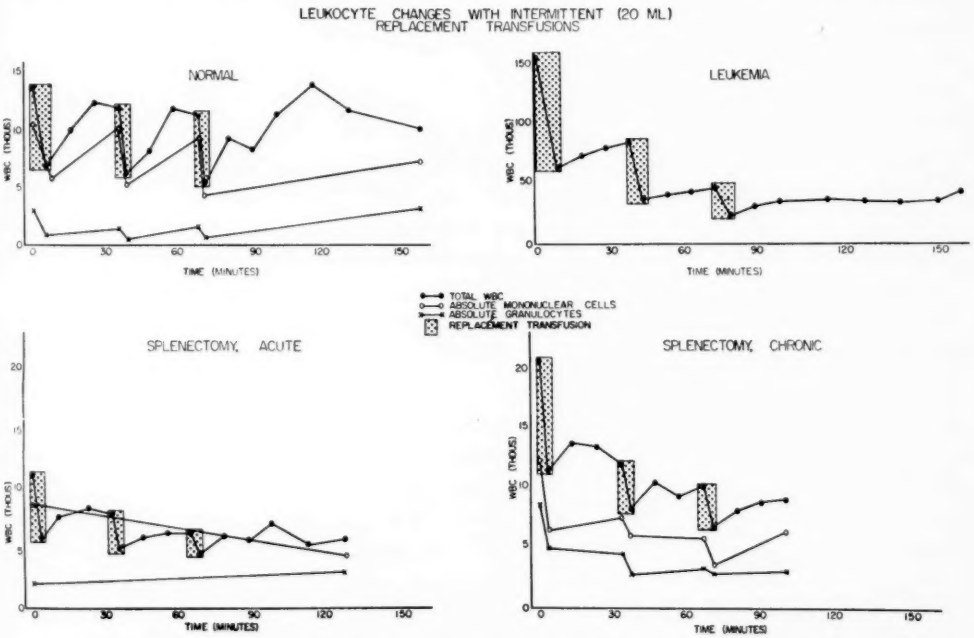

Fig. 3.—The mean total and differential leukocyte counts of 4 groups of rats during and after intermittent leukocyte replacement transfusions are shown above. Each stippled column represents the period during which a 20 ml. replacement transfusion was carried out.

the animals with the lower total leukocyte counts. These 2 rats had counts of 453,000 and 262,500 per cubic millimeter at the beginning of the experiment, and fell to 44,500 and 27,500 per cubic millimeter, respectively, after the last replacement, with very little change during the final 90-minute observation period.

In all types of experiments leukocyte counts were performed on the pooled blood obtained from the animals during replacement transfusion. The count of this blood was always approximately the mean of the count before and after replacement. It was evident, therefore, that reductions in leukocyte counts were entirely due to leukocyte removal, and did not represent sequestration of leukocytes within the animal.

DISCUSSION

These experiments were designed primarily to study the readily available leukocyte reserves of the normal rat by means of leukocyte removal, but also to observe the effects of splenectomy on this "pool" of preformed leukocytes. Rats with granulocytic chloroleukemia* were investigated in order to compare the responses of these animals with leukocytic disease with the responses of normal rats. Any technique for leukocyte removal obviously involves a nonphysiologic procedure, but the differences noted between the groups of animals suggest that the blood replacement procedure used in these experiments provided a useful tool for comparative study of the groups of animals used. The rat leukocyte count has proved quite stable in many different types of experiments in this laboratory. Indeed, attempts to produce granulocytic leukocytosis in rats have been unsuccessful, except by splenectomy. The replacement transfusion technique was very rapid, allowing 80 per cent replacement with leukopenic blood in 1 to 4 minutes. The general results obtained with removal of 2 to 3 circulating leukocyte volumes with a single continuous large replacement transfusion (50 ml.) were similar to those obtained when one circulating leukocyte volume was removed intermittently by a series of smaller (20 ml.) replacement transfusions.

The normal rats exhibited remarkable uniformity of response. Even during the few minutes involved in the actual process of replacement transfusion, the animals mobilized considerable numbers of leukocytes so that marked leukopenia could not be induced. The counts recovered within 30 minutes to the base-line level. It was evident that our experiments had failed to exhaust the readily available leukocyte "pool."

Splenectomy performed shortly before leukocyte removal resulted in the development of moderate levels of leukopenia, and the leukocyte counts did not recover as rapidly as in the normal. This was noted particularly in the intermittent type of leukocyte removal. Splenectomy 3 to 5 days before the experiment induced a marked leukocytosis and granulocytosis in the animals, presumably with granulocytic hyperplasia of the marrow caused both by removal of the spleen[5] and by postsplenectomy activation of latent *Bartonella muris* infection. These animals also were markedly depleted of leukocytes by the replacement transfusion, and did not regain their level of leukocytosis. These experiments appear to confirm the role of the spleen as a storage organ for preformed blood cells, a role that has frequently been attributed to the spleen.[5] However, it should be pointed out that in these studies leukopenia could not be induced even with continuous replacement transfusion with 50 ml. of blood, a procedure calculated to reduce the leukocyte count by 97 per cent in an adynamic system. These observations are consistent with the speculation that the splenectomized rat is deficient in some leukocyte mobilizing factor, since some readily available preformed leukocyte stores were demonstrated in these animals. Favoring the hypothesis of the spleen as one storage

*We would like to thank Dr. J. M. Ruegsegger and Miss Mary Jane Sparks of Lederle Laboratories and Dr. Harry Shay and Mrs. Margaret Gruenstein of Temple University for their help in establishing our leukemia colony.

site for leukocytes, however, is the fact that rats splenectomized immediately before leukocyte replacement demonstrated impairment of leukocyte mobilization.

The ease with which the leukemic rats were depleted is evidence that the preformed and readily available "pool" of leukocytes in this type of experimental leukemia is not increased in proportion to the leukocyte count. A quantitative comparison with the normal rat is not possible, however. Even though the proportionate mobilization was decreased, the leukemic rats during the various experimental procedures mobilized quantitatively more cells than did the normal. However, the absolute quantitative aspects of the leukocyte reserves of the normal rats were not determined since no evident exhaustion of these stores was affected by the experiments. In the studies on the leukemic rats no attempt was made to study the late effects of replacement transfusion, although Bessis[6] has suggested that replacement transfusion is beneficial in treatment of acute leukemia in humans.

Of particular interest in these studies was the fact that the leukocytes mobilized during these acute experiments (except in the leukemic group) were all of mature form, and were of the same cell types found in the blood before the replacement transfusions were performed. Thus the normal rat, with a marked predominance of lymphocytes, responded to leukocyte replacement largely with lymphocyte mobilization. The chronically splenectomized rats, however, had a higher proportion of granulocytes initially and mobilized both granulocytes and lymphocytes in response to leukocyte removal. Although there was some individual variation in the type of cell mobilized, the mean responses of granulocytes and mononuclear cells, as depicted in Figs. 2 and 3, bear out the fact that mobilization of both cell types occurred. Although it is possible that leukocyte removal in these different groups of experimental animals elicited proportional and equally rapid release from individual granulocyte and lymphocyte stores, it seems more plausible that the leukocyte reserves of the rat consist of premixed granulocytes and lymphocytes in similar proportion to the admixture of cells in the circulating blood. In other studies from this laboratory, it was concluded that small blood vessels may act as a storage site for transfused leukocytes in the irradiated rat and it seems possible that small vessels may be the principal site of storage of readily available mature leukocytes. Cells stored in sluggish vascular channels might well be premixed and would be available for mobilization within minutes into active circulation. The localization of these sluggish vascular channels may be widespread in the body, but the studies of the splenectomized animal suggest that the vessels of the spleen form one portion, probably not of major importance, of the intravascular storage depot in the rat. The type of vascular network within the spleen would seem ideally suited as a storage depot for the preformed granulocytes and lymphocytes.

The hypothesis that the leukocyte reserves of the rat consist of premixed adult cells situated within sluggish vascular channels appears directly contrary to the conclusions of Craddock and his co-workers[2] that in dogs the reserves of leukocytes are from mature cells awaiting the stimulus for delivery

from the bone marrow. However, the bone marrow is rich in capillary network and this network may serve as a reservoir of varying importance in different animal species. The leukopheresis procedure of Craddock induced a lymphopenia presumably due to stress of the procedure rather than to lymphocyte removal, so that his studies actually were concerned only with granulocytic replacement. The marked leukopenia persisting for 2 to 3 hours that was induced by leukopheresis may represent either species difference or differences due to the techniques employed. Certainly, the studies of the dog yield little data on the readily available pool of lymphocytes, yet Osgood's observations[3] suggest that the store of mature lymphocytes in man is vast.

SUMMARY

1. Leukocyte removal was accomplished in rats by replacement transfusion of normal, splenectomized, and leukemic animals by normal rat blood rendered leukopenic by mechanical removal of leukocytes.

2. The normal rat mobilized leukocytes rapidly, so that severe leukopenia did not develop during the period of replacement transfusion and recovery to normal leukocyte levels occurred within 30 minutes.

3. Splenectomized rats, whether splenectomy was performed immediately before or 3 to 5 days before replacement transfusion, did not mobilize leukocytes as rapidly as the normal animal. It was evident that these animals had available leukocyte stores, however, since severe leukopenia was not induced.

4. Rats with Shay granulocytic chloroleukemia also had available leukocyte reserves, but marked decrease in leukocyte count was obtained by replacement transfusion. It was evident that the available leukocyte reserves in these animals were not increased in proportion to the circulating leukocyte level.

5. In all experiments the leukocytes mobilized as a result of sudden leukocyte removal were mature and of the same cell type as in the circulation of the animals. This suggested that the leukocyte reserves of the rat consist of a premixed population of granulocytes and lymphocytes, rather than individual granulocytic and lymphocytic storage depots.

6. It was suggested that the immediately available leukocyte reserves of the rat consist of both granulocytes and lymphocytes stored within vascular channels and that the splenic vascular bed constitutes only one portion of that leukocyte "pool."

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A STUDY OF THE PLASMA THROMBIN TIME

ROBERT T. S. JIM, M.D.*
ST. LOUIS, MO.

INTRODUCTION

THE conversion of fibrinogen to fibrin constitutes the third or final stage of blood coagulation. This conversion may be accomplished by the addition of thrombin to plasma in vitro. The time required for the formation of a plasma fibrin clot upon the addition of thrombin is defined as the plasma thrombin time. Prolongation of the plasma thrombin time has been reported in many diseases, including cirrhosis, metastatic cacinoma to the liver, multiple myeloma, severe pre-eclampsia, eclampsia, amniotic fluid embolism, disseminated lupus erythematosus, malabsorption syndrome, Waldenström's macroglobulinemia, and in the newborn.[1-10] However, the basic mechanisms underlying the prolonged plasma thrombin time are not well understood. The conversion of purified preparations of fibrinogen to fibrin by thrombin is influenced by the temperature, pH, calcium concentration, and colloidal state of the substrate.[11] Platelet extracts (platelet factor 2) have been shown to accelerate the interaction of purified preparations of fibrinogen and thrombin.[12] The thrombin time of normal heparinized plasma increases upon storage, presumably due to increased heparinoid activity[13] and may be shortened by the addition of serum or plasma pseudoglobulin.[14] Increase in plasma antithrombin may be a contributing factor. Pronounced plasma hypofibrinogenemia appears to be an infrequent cause for a delayed thrombin time. The abnormal plasma proteins in multiple myeloma may occasionally interfere with or inhibit the conversion of fibrinogen to fibrin.[1, 2, 5, 7] Fibrinogen itself may be qualitatively abnormal and less reactive to the clotting action of thrombin.[8-10, 15-17] Deficiency of a plasma factor which accelerates the conversion of fibrinogen to fibrin has been recently postulated.[6]

Because the mechanisms responsible for prolongation of the plasma thrombin time are not completely understood, a study of the plasma thrombin time was carried out in a variety of diseases. The prolonged plasma thrombin times in this study were found to be due to a deficiency of plasma "albumin," elevation of the plasma fibrinogen level, and, in rare instances, circulating anticoagulants.

MATERIALS AND METHODS

Techniques employed for the routine coagulation studies have been published elsewhere.[9] Whole blood was anticoagulated with 0.1 M sodium oxalate (1 part oxalate to 9 parts blood) or with dried balanced oxalate. Plasma was defibrinated with bovine thrombin† and allowed

From the Department of Internal Medicine, Washington University School of Medicine, St. Louis, Mo.
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to stand several hours before use. The subsequent addition of bovine fibrinogen to this plasma failed to induce a clot. Bovine thrombin was dissolved in veronal buffer[6] and stored frozen in small aliquots or kept at 4° C. in 50 per cent glycerol. Bovine fibrinogen* was dissolved in saline and used fresh. Fibrinogen concentrations were determined by the method of Ratnoff (normal range, 164 to 415 mg. per cent).[18] Normal or the patient's serum was decalcified with 0.1 M sodium oxalate (1 part oxalate to 9 parts serum).

Plasma Thrombin Time.—The plasma thrombin time was performed by determining the time necessary for the coagulation of 0.4 ml. plasma (anticoagulant: dried balanced oxalate) after 0.1 ml. thrombin (frozen, 3.3 μ/ml.) was added.

Antithrombin Activity.—Antithrombin activity was determined by measuring the ability of 24-hour-old serum (kept in sterile glass tubes at 37° C.) to inactivate a standard quantity of thrombin.

Isolation of Fibrinogen.—Fibrinogen was isolated from plasma by precipitation in phosphate buffer[19] or by the freeze-thaw technique.[20] The absorption of plasma with $Al(OH)_3$ was omitted prior to the phosphate buffer precipitation and the final fibrinogen product was dialyzed for 8 hours. Fibrinogen obtained from 6 ml. of plasma by the freeze-thaw technique was washed 3 times with cold saline and reconstituted in 2 ml. saline.

Ammonium Sulfate Fractionation.—Normal defibrinated plasma† or decalcified serum, both absorbed with barium sulfate, was dialyzed for 48 hours at 4° C. against 33 per cent, then 60 per cent saturated ammonium sulfate solutions. The precipitates were separation by centrifugation, washed 4 times with the respective ammonium sulfate solutions, reconstituted in saline to their original volumes and dialyzed against saline at 4° C. for 48 hours. The supernatants of the 60 per cent ammonium sulfate precipitates were dialyzed against saline in a similar manner.

Viscosity Index.—All viscosity studies were carried out in an Ostwald viscosimeter. The plasma viscosity was determined on a 10 ml. sample (anticoagulant: dried balanced oxalate) at 27.5° C.; the viscosity, compared to 10 ml. distilled water, was expressed as the "viscosity index."

RESULTS

A total of 88 patients were studied. The majority of patients were selected at random; however, toward the latter part of the study patients were selected with hypoalbuminemia.

Plasma Thrombin Time.—The normal plasma thrombin times ranged from 26 to 32 seconds. Prolonged plasma thrombin times were found in nearly all of the diseases studied, including 2 patients in late pregnancy and in 3 out of 4 newborns. Included in the study were hepatocellular liver diseases of many etiologies, acute and chronic leukemias, malignant lymphomas, malignancies, infections, multiple myeloma, collagen diseases, granulomas, metabolic disorders, malnutrition, and renal diseases. The thrombin times were usually increased from 1½ to 2 times over the normal control, but were occasionally prolonged more than 3 to 4 times. Normal thrombin times were encountered in a few patients. No correlation was noted between the thrombin time, platelet count, whole blood clotting time, or plasma prothrombin time.

Fibrinogen Concentration.—The fibrinogen concentrations of the patients ranged from 220 to 1,178 mg. per cent. Over half of the patients exhibited moderately elevated fibrinogen concentrations. Normal fibrinogen concentrations were found in the plasmas of the newborn and slightly elevated levels were obtained on women late in pregnancy. In no instance was hypofibrinogenemia

*The Armour Laboratories, Chicago, Illinois.

†Fibrinogen was removed from plasma by the addition of thrombin.

observed. Although a wide range of plasma thrombin times was found at any one fibrinogen concentration, an increase in plasma thrombin times could be correlated roughly with increase in fibrinogen concentration (Fig. 1). Many plasma thrombin times were prolonged in the presence of normal fibrinogen concentrations, but at no time were normal thrombin times observed in the presence of elevated fibrinogen levels.

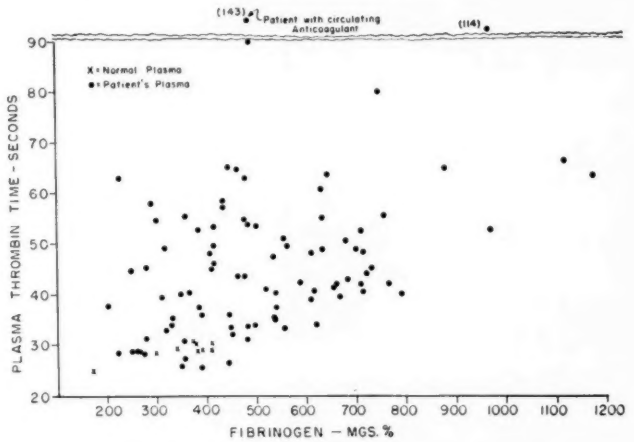

Fig. 1.—The relationship of the plasma thrombin time to fibrinogen concentration. With increasing plasma fibrinogen concentration, there is a tendency toward a corresponding rise in plasma thrombin time ($r = 0.29$, $p < 0.01$).

Antithrombin Activity.—Essentially normal antithrombin activity was found in most of the sera examined, but some exhibited moderate increases or decreases (Fig. 2). Two sera with increased antithrombin activity were from patients receiving Dicumarol. The plasmas of patients with increased antithrombin activity did not demonstrate greater prolongation of their thrombin times than the plasmas of patients associated with normal antithrombin activity. In the presence of decreased antithrombin activity the plasma thrombin times were still prolonged.

Plasma Viscosity.—Increased plasma viscosity was found in many of the patients studied and correlated better with elevation of the fibrinogen than with the plasma globulin level. In the majority of plasmas the increase in viscosity was associated with elevation of both the plasma fibrinogen and globulin, but in a few, with either hyperfibrinogenemia or hyperglobulinemia alone. Normal or only slightly increased fibrinogen or globulin concentrations were found in plasmas with normal viscosities. In no instance was elevation of the plasma viscosity associated with both normal fibrinogen and globulin concentrations. In general, the plasma viscosity observed in diseases other than multiple myeloma was slightly to moderately elevated, while more pronounced increases in viscosity were found in multiple myeloma and in a patient with lymphosarcoma; in the latter instances, the abnormal globulins demonstrated in the plasma were characterized by narrow, tall, sharp gamma, "M" or beta globulin peaks on electrophoresis.

A wide range of plasma thrombin times was found at any one viscosity index. Although a correlation was suggested between the slight to moderate increase in plasma viscosity and prolongation of the thrombin time (Fig. 3), extreme increases in plasma viscosities observed in patients with multiple myeloma and in the patient with lymphosarcoma were not accompanied by proportional increases in the thrombin times. Many plasmas with prolonged thrombin

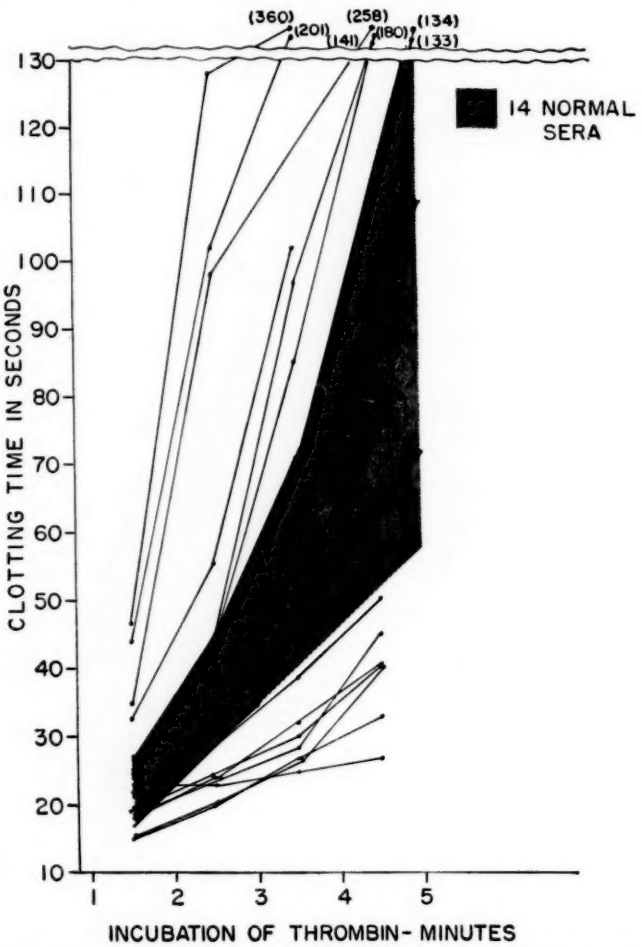

Fig. 2.—Antithrombin activity. 0.8 ml. thrombin (6.6 μ/ml.) was incubated with 0.8 ml. of the patient's serum and, at the intervals indicated, 0.4 ml. of the mixture was withdrawn and added to 0.2 ml. bovine fibrinogen (300 mg. per cent) and the clotting time was then determined. Normal antithrombin activity is present in the majority of sera with few demonstrating increased or decreased activity.

times were found to have normal viscosities, yet their thrombin times were improved by the addition of normal defibrinated plasma. Normal plasma thrombin times were not observed in the presence of abnormally high plasma viscosities. Normal plasma viscosities were found in the 2 patients in late pregnancy and in the 4 newborns.

In vitro, a correlation between increasing viscosity and prolongation of the clotting of fibrinogen by thrombin could be demonstrated by the addition of

polyvinylpyrrolidone or increasing concentrations of isolated bovine or human fibrinogen (Fig. 4, *A* to *C*). In a patient with multiple myeloma, a reduction in temperature resulted in marked increase in the plasma viscosity and corresponding increase in the plasma thrombin time of greater degree than that observed in the normal control plasma (Fig. 4, *D*).

Demonstration of Normal Clotting Reactivity of the Isolated Fibrinogen.—Fibrinogen isolated from pathologic plasmas with prolonged thrombin times clotted as readily as that obtained from normal plasmas upon the addition of thrombin (Fig. 5). As the concentration of the patient's or normal isolated fibrinogen increased, there was a gradual prolongation of the clotting time of the fibrinogen by thrombin; with decreasing concentration of the isolated fibrinogen below a 100 mg. per cent, a more abrupt increase in clotting time of the fibrinogens by thrombin resulted. The fibrinogen from an occasional plasma failed to precipitate out by the freeze-thaw method. Qualitative abnormalities could not be excluded in the fibrinogens of these patients. The isolated fibrinogen of only one of the four plasmas from newborns was studied and found to be normal (the thrombin time of this newborn plasma was normal).

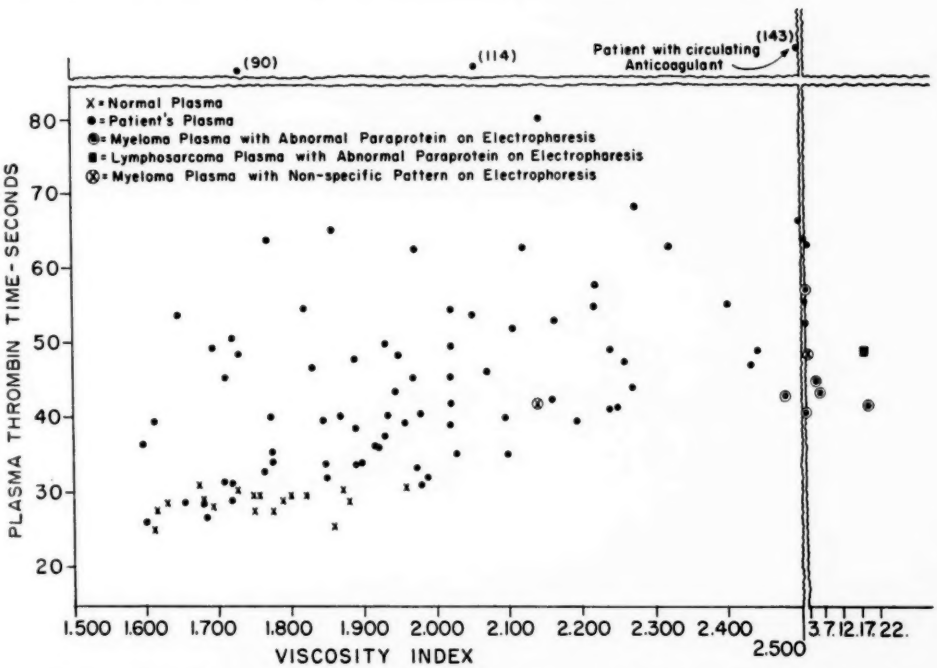

Fig. 3.—The relationship of plasma thrombin time and plasma viscosity. A correlation is suggested between the plasma thrombin time and slight increases in plasma viscosity. However, extreme elevation in viscosity is not accompanied by proportional rise in thrombin time. No over-all correlation was found between the plasma thrombin time and viscosity on statistical analysis.

Failure of Normal Platelet Suspensions to Correct the Prolonged Plasma Thrombin Time.—The addition of normal platelet suspensions to the patient's plasma in equal volumes failed to correct the prolonged thrombin time of the latter.

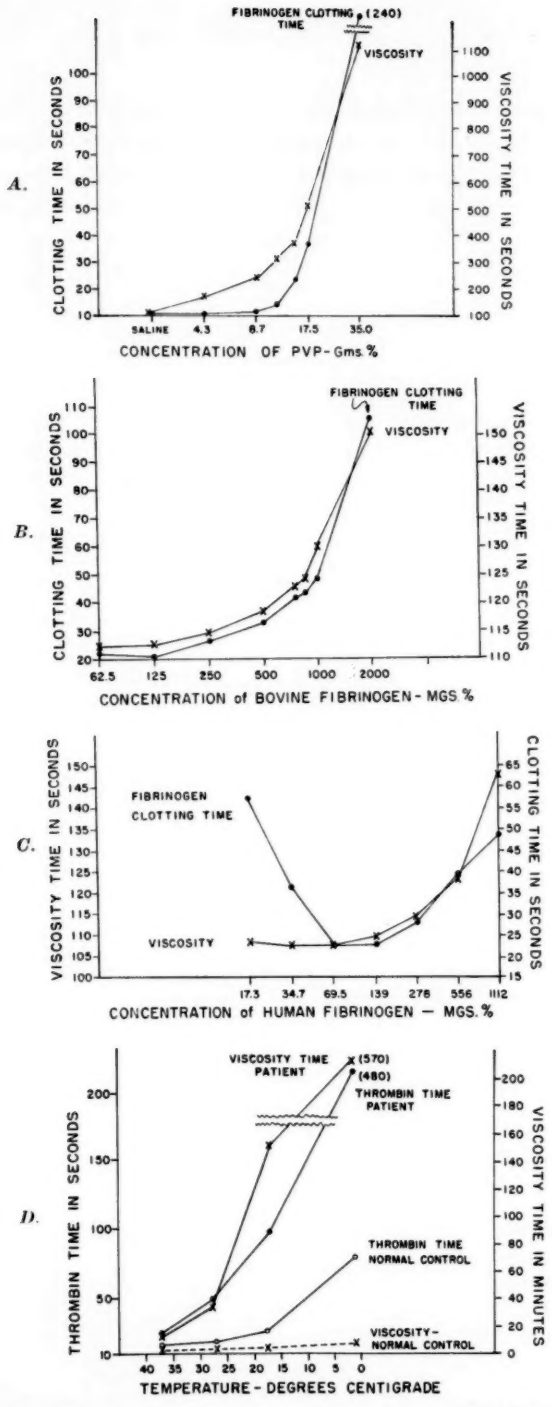

Fig. 4.—The influence of increasing viscosity on the bovine fibrinogen and plasma thrombin times. *A*, The effect of increasing concentration of polyvinylpyrrolidone (PVP) on the viscosity and clotting time of a PVP-bovine fibrinogen mixture. To 0.2 ml. bovine fibrinogen (300 mg. per cent) and 0.2 ml. PVP dissolved in distilled water in the concentration indicated, 0.1 ml. thrombin (6.6 μ/ml.) was added. The viscosity of 5 ml. of the mixture was determined at 25° C. *B*, The effect of increasing concentration of bovine fibrinogen on the viscosity and thrombin clotting time. The 0.4 ml. bovine fibrinogen dissolved in saline in the concentration indicated, 0.1 ml. thrombin was added as above. Viscosity was determined as in *A*. *C*, The effect of increasing concentration of human fibrinogen on the viscosity and thrombin clotting time. Human fibrinogen was isolated from normal plasma by the freeze-thaw technique and studied in similar manner as *B*. *D*, The effect of decreasing temperature on the plasma thrombin time and viscosity in a patient with myeloma.

Effect of Dilution of Plasma on the Prolonged Thrombin Time.—Progressive serial dilution of the plasmas with saline to 1:64 generally led to a gradual prolongation of the plasma thrombin times (Fig. 6). A transient shortening of the thrombin time was associated with the plasmas exhibiting higher fibrinogen levels while the dilution curves of plasmas with more normal fibrinogen levels were similar to the dilution curves of the normal control plasmas. This phenomenon could be reproduced in vitro by varying the fibrinogen level of the plasma to be diluted. Normal defibrinated plasma to which was added bovine fibrinogen to a concentration of 2,000 mg. per cent showed transient shortening of the thrombin time on dilution with saline. A bovine fibrinogen level of 200 mg. per cent in the normal defibrinated plasma resulted in a more normal type of dilution curve. In one patient with far-advanced cirrhosis, marked shortening of the prolonged plasma thrombin time occurred upon dilution of his plasma despite only slight elevation of the fibrinogen level (492 mg. per cent) (Fig. 6).

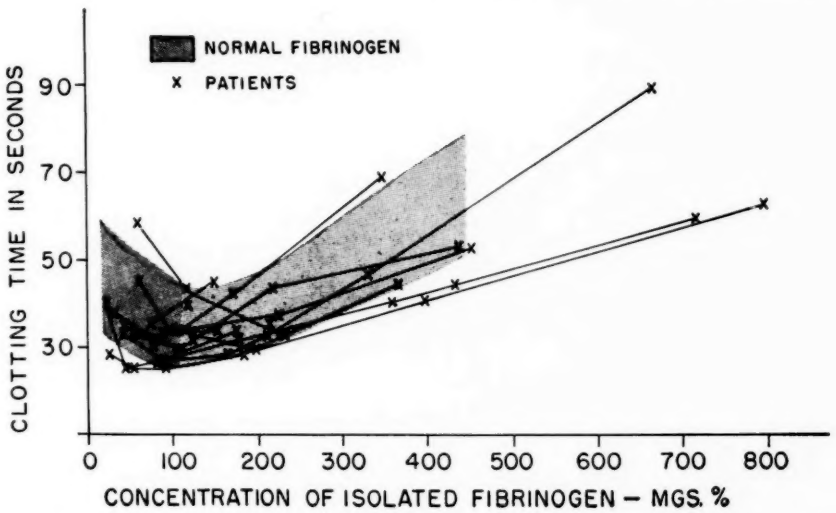

Fig. 5.—Demonstration of normal clotting reactivity of the isolated fibrinogen. To 0.4 ml. of the isolated fibrinogen dissolved in saline 0.1 ml. thrombin (1.1 μ/ml.) was added and the clotting time determined. The fibrinogens obtained from pathologic plasmas clotted as readily as those obtained from normal plasmas.

In this patient, the plasma formed a jellylike clot upon the addition of thrombin, the plasma viscosity was slightly increased (viscosity index 2.514), the antithrombin activity decreased, serum globulin elevated (6.2 Gm. per cent), and the whole blood clotting time prolonged. The addition of 1 part of the patient's plasma to 9 parts of normal plasma resulted in prolongation of the thrombin time of the latter. A circulating anticoagulant was felt to be present in this patient inhibiting the conversion of fibrinogen to fibrin.

The Corrective Effect of Normal Whole or Defibrinated Plasma or Serum on the Prolonged Plasma Thrombin Time.—

The addition of normal whole or defibrinated plasma: The addition of 1 part of the patient's whole or defibrinated plasma to 9 parts of normal plasma

did not significantly prolong the thrombin time of normal plasma over that produced by the addition of saline. One part of normal whole plasma consistently improved the prolonged plasma thrombin time of 9 parts of the patient's plasma.

The addition of normal defibrinated plasma in equal or one-tenth the volume amounts to the patient's plasma resulted in significant reduction in the prolonged thrombin time in almost all of the plasmas studied, irrespective of the disease

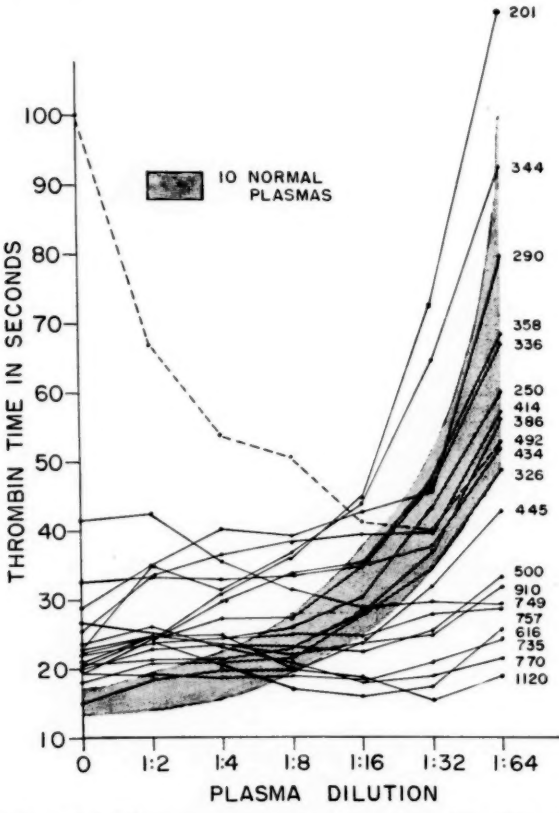

Fig. 6.—Effect of dilution on the prolonged plasma thrombin time. To 0.4 ml. of the patient's plasma (anticoagulant: 0.1 M sodium oxalate) serially diluted with saline to 1:64, 0.1 ml. thrombin (6.6 μ/ml.) was added and the clotting time determined. The number to the right of each curve represents the fibrinogen concentration of the undiluted plasma. The plasma with the circulating anticoagulant is indicated in the broken line.

process (Fig. 7). The effect was more pronounced when equal volumes of normal defibrinated plasma were employed. In most instances the abnormal thrombin time did not quite return to normal, although some plasmas with only slight prolongation of the thrombin times returned completely to normal. In contrast the addition of an equal volume of saline resulted in further increase in the majority of the samples. The mixture of equal amounts of (a) two abnormal whole plasmas with prolonged thrombin times, (b) one abnormal plasma defibrinated and another whole plasma with a prolonged thrombin time, or (c) a pathologic plasma defibrinated and its own whole plasma failed to correct each other, although all were shortened separately by the addition of normal defibrinated plasma. Normal plasma defibrinated by heating at 56° C. for 3 minutes

still possessed corrective effect. The prolonged thrombin times of 2 of the 3 newborn cord plasmas were not shortened by the addition of equal amounts of normal defibrinated plasma, but in the third newborn there was improvement. The slightly prolonged plasma thrombin times in late pregnancy were normalized by the addition of normal defibrinated plasma.

The addition of normal serum: Normal serum in equal or one-tenth the volume amounts produced even greater shortening than comparable quantities of normal defibrinated plasma, returning many of the abnormal thrombin times to normal (Fig. 7). The accelerating effect of serum was partially diminished by incomplete decalcification with sodium oxalate (1 part oxalate to 9 parts serum). After decalcification, the accelerating effect of serum approximated that of normal defibrinated plasma. Normal serum produced a slight shortening of the normal plasma thrombin time, but the effect could be eliminated by decalcification. Decalcified sera obtained from patients with prolonged plasma thrombin times exhibited little or no accelerating effect on plasmas with prolonged thrombin times.

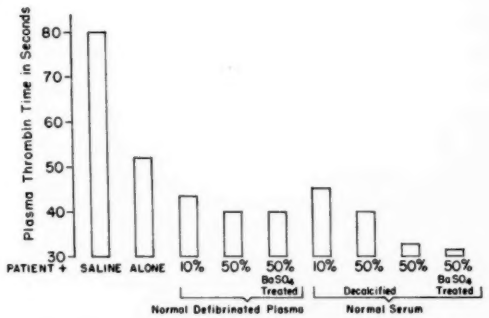

Fig. 7.—The corrective effect of normal defibrinated plasma and serum on the prolonged plasma thrombin time. The values indicated represented the average of 26 patients studied. The various reagents indicated along the abscissa were added to the patient's plasma in equal or one-tenth the volume amounts. To 0.4 ml. of the mixture or patient's plasma, 0.1 ml. thrombin (3.3 μ/ml.) was added and the clotting time determined. The normal control thrombin time ranged from 26 to 32 seconds.

Physicochemical study of the accelerator under investigation: The accelerator effect in normal defibrinated plasma or serum was not decreased by dialysis, barium sulfate absorption, freezing, prior removal of platelets from plasma by centrifugation, factor V depletion (by heating at 37° C. for 24 hours), or Dicumarol administration. Of the 2 newborn cord plasmas studied both showed no reduction in the accelerator effect.

The thrombin time of normal whole plasma was not influenced by barium sulfate absorption, dialysis, removal of platelets by centrifugation, clotting in glass or siliconized tubes, or by Dicumarol administration. The addition of an equal volume of normal defibrinated plasma to whole normal plasma did not shorten the thrombin time of the latter; the result excludes the possible activation in normal plasma of an accelerator substance by thrombin. Mixtures of equal volumes of normal plasmas gave values no shorter than each tested separately, thereby excluding the possibility of nonspecific acceleration of the thrombin time due to mixtures of foreign plasmas.

Plasmas from patients deficient in antihemophilic globulin, plasma thromboplastin component, plasma thromboplastin antecedent, Hageman trait, and from a patient with agammaglobulinemia exhibited normal thrombin times and no reduction in the accelerator effect when tested on pathological plasmas with delayed thrombin times.

Heat Stability.—The thrombin time of normal or pathologic plasmas increased only slightly when the plasma stood at room temperature for 9 hours. Normal sterile plasmas incubated in glass tubes at 37° C. displayed a gradual moderate increase in thrombin times after many days' storage. The accelerator effect was still demonstrable in normal plasma after storage at 37° C. for 77 days and in normal decalcified serum kept at 4° C. for 56 days. Heating normal defibrinated, barium sulfate-treated plasma or decalcified serum at 60° C. for up to 60 minutes resulted in gradual loss of activity when tested on the pathologic plasmas; the heated normal defibrinated plasma when added to normal plasma in equal amounts actually resulted in increase in the thrombin time of the latter.

Ammonium Sulfate Fractionation.—Normal defibrinated plasma and decalcified serum, both absorbed with barium sulfate, were subjected to ammonium sulfate fractionation. The 0 to 33 per cent ammonium sulfate precipitates obtained from plasma or serum demonstrated no accelerator effect, the 33 to 60 per cent precipitates revealed slight accelerator effect, and the supernatant of the 33 to 60 per cent precipitates showed maximal accelerator effect. None of the plasma or serum ammonium sulfate precipitates contained significant thrombin activity. Thrombin added to these precipitates produced no fibrin clots. On paper electrophoresis the 0 to 33 per cent precipitates contained gamma globulin only; the 33 to 60 per cent precipitates, mainly gamma, beta, and alpha globulins with 6.9 to 8.5 per cent albumin; the supernatant of the 33 to 60 per cent precipitates had approximately 84 to 94 per cent albumin with small amounts of alpha and beta globulins.

Plasma from a patient with cirrhosis and a moderately prolonged plasma thrombin time was similarly subjected to ammonium sulfate fractionation. No accelerator effect was found in the 0 to 33 per cent and 33 to 60 per cent precipitates, and markedly diminished accelerator effect in the supernatant of the 33 to 60 per cent precipitate. On paper electrophoresis, similar electrophoretic components were found in the various ammonium sulfate precipitates and supernatant, with an increase in all the globulin fractions and a marked decrease in the albumin fraction.

The supernatant of the 33 to 60 per cent ammonium sulfate precipitates of normal plasma and serum exhibited moderate loss of activity after they were heated at 60° C. for 30 minutes.

Plasmas from one patient with disseminated lupus erythematosus and from one with malnutrition (serum albumin 1.6 and 1.7 Gm. per cent, respectively) were subjected to similar barium sulfate absorption and ammonium sulfate fractionation. The supernatants of the 60 per cent precipitates, containing mainly albumin, were lyophilized and reconstituted in smaller volumes of saline approximating the albumin concentration of the normal control (serum albumin 4.3 Gm. per cent). The concentrated albumin preparations from the patients

exhibited some accelerator effect but to a lesser degree than the normal control albumin. Although concentration of the albumin had been achieved, the effect was partly abolished by the concomitant increase in antithrombin activity in the albumin preparation.

*Cohn's Plasma Fractions.**—Cohn's plasma fractions were dissolved in saline in 3 Gm. per cent concentration and added to the pathologic plasmas in equal volumes. The accelerator effect was found mainly in fraction V with slight activity in fraction IV-4. No activity was found in fractions I, II, III, IV-1, and IV-7. Actually, fractions I, II, and III demonstrated an inhibitory effect on the thrombin time of the pathological plasmas. On paper electrophoresis, fraction V contained almost 100 per cent albumin; fraction IV-4, 33 per cent albumin with smaller amounts of alpha, beta, and gamma globulins; and fraction IV-1, only trace amounts of albumin with larger amounts of alpha, beta, and gamma globulins. Albumin was not present in detectable amounts in the remaining Cohn fractions.

Electrophoretic Separation of Normal Plasma and Serum.—By the technique of paper electrophoretic separation, the accelerator effect in normal defibrinated, barium sulfate-treated plasma and normal decalcified serum was localized mainly in the albumin component with slight accelerator effect noticeable in the alpha-1 and gamma-1 globulins.

The Accelerator Effect of Transfused Normal Whole Blood in Vivo.—The infusion of one unit of normal whole blood (17 days old, kept at 4° C.) to a patient with aplastic anemia and hypoalbuminemia resulted in significant improvement in the prolonged plasma thrombin time over a period of 4 days during which observations were made.

The Effect of Commercial Gamma Globulin, Bovine and Human Albumin, and Acacia on the Plasma Thrombin Time.—

Gamma globulin: Gamma globulin (immune human globulin) in 1 Gm. per cent concentration displayed no accelerator effect on the prolonged plasma thrombin time in vitro.

Albumin: Bovine† or human albumin in 4.25 Gm. per cent concentrations demonstrated even more marked accelerator effect than normal defibrinated plasma in vitro. The effect was not reduced by decalcification with oxalate, but diminished to a slight extent when the preparations were heated at 60° C. for 30 minutes. The infusion of 100 ml. of a 25 per cent concentration human salt-poor albumin‡ into one patient with severe cirrhosis, hypoalbuminemia (serum albumin 1.5 Gm. per cent), and marked ascites and into another patient in the nephrotic phase of chronic glomerulonephritis (serum albumin 2.0 Gm. per cent) with anasarca, resulted in impressive though transient improvement in the prolonged plasma thrombin times (Fig. 8).

Acacia: Acacia in varying concentrations from 0.56 to 4.5 Gm. per cent exhibited more pronounced accelerator effect in vitro than comparable quantities

*Supplied by the American Natonal Red Cross, National Headquarters, Washington, D. C., through the courtesy of Dr. J. N. Ashworth.

†The Armour Laboratories, Chicago, Ill.

‡Courtland Laboratory, Los Angeles, Calif.

of normal defibrinated plasma on the prolonged plasma thrombin time. Shortening of the thrombin time also occurred when acacia was added to normal plasma. Heating acacia at 60° C. for 30 minutes or decalcification with sodium oxalate resulted in slight loss of accelerator effect.

The Lack of Corrective Effect of Normal Defibrinated Plasma Made Hypoalbuminemic by Saline on the Prolonged Plasma Thrombin Time.—Normal defibrinated plasma made hypoalbuminemic by dilution with saline (serum albumin 1.4 Gm. per cent) was as ineffective in improving the prolonged plasma thrombin time as plasma from a patient with cirrhosis and a serum albumin concentration of 1.7 Gm. per cent.

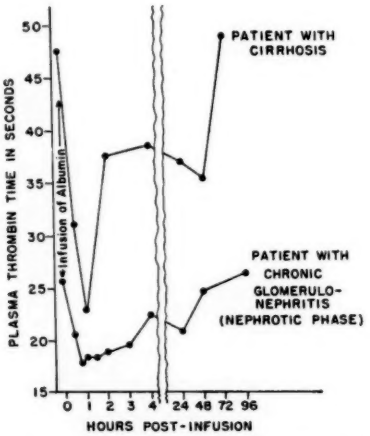

Fig. 8.—The in vivo corrective effect of intravenous infusions of human albumin on the prolonged plasma thrombin time. 100 ml. of 25 per cent human salt-poor albumin was administered intravenously over a 20-minute period and the plasma thrombin times (0.1 ml. glycerol-thrombin, 5 μ/ml., added to 0.4 ml. plasma) determined at the intervals indicated following the infusions. Note the rapid, but transient corrective effect of the infused albumin on the prolonged plasma thrombin times of the patients.

Correlation of the Prolonged Plasma Thrombin and Bovine Fibrinogen Clotting Times With the Serum Albumin Level.—The increase in plasma thrombin time could be correlated with the serum albumin level. The patients with normal or slightly decreased serum albumin levels demonstrated normal or slightly prolonged plasma thrombin times. With decreasing serum albumin levels, the plasma thrombin times became increasingly prolonged (Fig. 9, *A*). No correlation was evident between the plasma thrombin times and serum globulin levels.

The clotting time on the addition of thrombin to a mixture of bovine fibrinogen with the patient's defibrinated plasma or bovine albumin could be correlated with the albumin level of the mixture. Depression of the albumin level resulted in prolongation of the bovine fibrinogen clotting time (Fig. 9, *B*).

DISCUSSION

The results of this study confirm many of the previous observations in which prolonged plasma thrombin times were reported in a variety of disease states. Prolongation of the plasma thrombin time appeared to be due to a multiplicity of factors, often occurring in combination. The most constant abnormality appeared to be a deficiency of a substance in plasma aiding the conversion of

fibrinogen to fibrin. In addition, an increase in fibrinogen concentration also seemed to contribute to the delayed thrombin time in many of these patients. Hypofibrinogenemia was not observed in the present series. The antithrombin activity did not generally seem to correlate with the prolonged plasma thrombin time although increases in antithrombin were found in a few patients and may

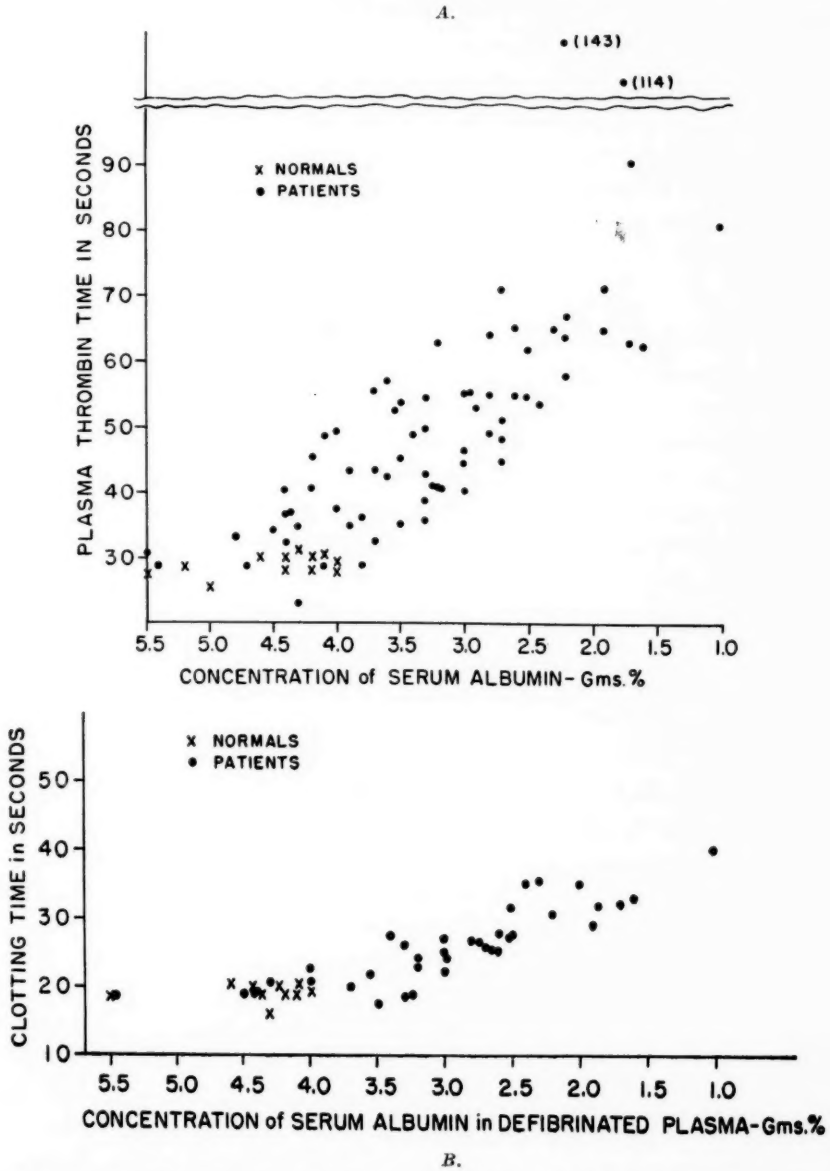

Fig. 9.—The effect of the concentration of albumin on the plasma thrombin and bovine fibrinogen clotting times. *A*, The effect of decreasing serum albumin concentration on the plasma thrombin time. With decreasing serum albumin concentration, note the gradual increase in plasma thrombin time. *B*, The effect of decreasing albumin concentration of the patient's defibrinated plasma on the bovine fibrinogen clotting time. To a mixture of 0.3 ml. of the patient's defibrinated plasma (anticoagulant: dried balanced oxalate) and 0.1 ml. bovine fibrinogen (300 mg. per cent), 0.1 ml. thrombin (6.6 μ/ml.) was added and the clotting time determined. With decreasing serum albumin concentration of the patient"s defibrinated plasma, note the gradual increase in bovine fibrinogen clotting time.

have contributed to the abnormality. Only a single instance of circulating anticoagulant was recognized. Abnormal clotting reactivity of fibrinogen to thrombin could not be demonstrated in the plasmas studied.

The data presented are in agreement and confirm all observations previously reported on a fibrinogen conversion accelerating effect in normal defibrinated plasma. In addition, the accelerator effect was also demonstrated in normal serum. The greater accelerator effect of serum than plasma is probably due to the known clot-accelerating effect of calcium in serum.[21] The studies in this report also suggest no relationship between platelets or any of the known plasma or serum coagulation factors and the accelerator effect in plasma or serum. Suspensions of normal platelets were shown to possess no accelerator effect on the prolonged plasma thrombin times. No correlation was evident between the platelet count and thrombin time.

The data obtained indicate that the accelerator effect in normal plasma or serum was associated mainly with albumin or a substance related to albumin. The apparent localization of the accelerator effect to albumin was supported by many observations in this study. The increase in plasma and bovine fibrinogen clotting times correlated well with decreasing serum albumin levels; no correlation was evident with serum globulin levels. The ammonium sulfate fractionated precipitates of normal plasma or serum containing the active accelerator effect were associated with the albumin component. While the fractions of normal plasma with maximal activity were different from that previously reported,[6] the difference may have been in the technique of fractionation. By paper electrophoretic separation, the accelerator effect in plasma and serum was confined mainly to the albumin. Pure bovine or human albumin preparations exhibited accelerator effect in vitro and with the latter preparation, also in vivo. The accelerator effect was found primarily in Cohn's plasma fraction V, which contained approximately 99 per cent albumin, and to a slight extent in fraction IV-4, which contained in addition to alpha and beta globulins, 32.9 per cent albumin.

Whether the accelerator effect in albumin is due to a true coagulation factor or to a nonspecific colloidal effect was not resolved. The conversion of fibrinogen to fibrin in vitro is dependent on an optimal colloidal state.[11] Acacia in 2 to 6 per cent concentration can accelerate the conversion of fibrinogen to fibrin probably by providing a more optimal colloidal state. In this report acacia demonstrated a similar accelerating effect in vitro on plasmas with delayed thrombin times. The almost universal tendency for the serum albumin level to fall with acute or chronic disease correlates well with the occurrence of increased plasma thrombin times in many disease processes. The decreased serum albumin levels may result in suboptimal colloidal states. Accelerator effect in normal defibrinated plasma and serum probably acts simply by restoring a suboptimal colloidal state to normal. The moderate loss of accelerator effect in normal defibrinated plasma or decalcified serum after heating could be due

to destruction of a coagulation factor in albumin, to denaturation of the albumin molecule with impairment of its colloidal effect or to loss of antiheparin activity in pseudoglobulin.[14] The heat stability of the accelerator effect of normal plasma in this report was greater than in the one previous study.[6]

Although the influence of fibrinogen concentration on the clotting time of isolated thrombin-fibrinogen mixtures has been well studied, previous work has not emphasized hyperfibrinogenemia as a contributing factor in prolongation of the plasma thrombin time. The present data are in accord with the known tendency of increased concentrations of fibrinogen to clot more slowly. The associated rise in viscosity with increasing concentrations of fibrinogen, whether in plasma or in isolated form, may represent an associated secondary phenomenon rather than a direct causative factor in prolongation of the fibrinogen clotting time. While prolongation of the plasma thrombin time by polyvinylpyrrolidone was observed in this study, shortening of the thrombin time by this substance has been reported previously.[22] In addition, the plasma thrombin time in patients with multiple myeloma showed no direct proportional increase with the marked rise in plasma viscosity observed, but seemed to correlate better with the fibrinogen level. An excess of fibrinogen molecules might mechanically interfere with the process of fibrinogen polymerization and fibrin clot formation.

In late pregnancy the slight increase in plasma thrombin time appears to be due to a reduction in albumin and some increase in fibrinogen concentration. The number of newborn plasmas studied in this report is too small to permit definite conclusions, but reduction in serum albumin and possible qualitative changes in the fibrinogen may be important factors in the prolongation of the plasma thrombin time.

Most of the abnormal thrombin times were associated with normal blood coagulation times. No correlation was found between the coagulation time and abnormal thrombin time. In the few patients with prolonged whole blood coagulation times, the thrombin times were not excessively prolonged with one exception. The relative lack of prolongation of whole blood coagulation in the presence of increased thrombin time may be due to the relative shortness of the third stage of coagulation compared to over-all coagulation.

The increase in plasma thrombin times did not appear to result in serious hemorrhagic tendencies in the patients studied. The majority of patients were free from clinical bleeding at the time of study. In most of the patients with hemorrhagic manifestations, the thrombin times were not excessively prolonged and other coagulation defects were usually present. The wide spectrum of diseases in which the plasma thrombin time may be prolonged and the relative lack of associated hemorrhagic tendencies in the great majority would suggest the increased plasma thrombin time to have limited clinical significance.

SUMMARY

Prolongation of the plasma thrombin time was found in a wide variety of diseases and in late pregnancy and the newborn. Further study revealed the increase in plasma thrombin time to be a complex phenomenon due to a multiplicity of factors, usually occurring in combination. The most constant abnormality appeared to be a deficiency of a plasma substance aiding the conversion

of fibrinogen to fibrin. An increase in plasma fibrinogen appeared to be a contributing factor. Circulating anticoagulant was encountered only once and increased antithrombin activity appeared to play a relatively insignificant role in the delayed plasma thrombin time. The deficient substance in plasma, aiding fibrin clot formation, did not appear to be platelets or any of the known plasma or serum coagulation factors. The data presented suggest that the deficient substance is albumin or a substance related to albumin. Clinically, prolongation of the plasma thrombin time did not appear to result in significant hemorrhagic manifestations.

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THE ROLE OF FACTOR V IN THE FORMATION OF BLOOD THROMBOPLASTIN

CECIL HOUGIE, M.B., B.S. (LOND.), M.R.C.S. (ENG.)*
CHARLOTTESVILLE, VA.

BIGGS, Douglas, and Macfarlane[1] showed that the incubation of $Al(OH)_3$-adsorbed plasma, serum, platelets, and calcium results in the formation of a powerful coagulant which they named "blood thromboplastin." The term "thromboplastin generation" implies the formation of a final prothrombin converting substance; while this may not necessarily be true, it, nevertheless, forms a useful working hypothesis. There are, however, other interpretations of the phenomenon. For example, Seegers and his associates[2, 3] believe that the thromboplastin generation test measures the transformation of plasma AC-globulin (factor V, proaccelerin) to serum AC-globulin.

It was originally believed by Biggs, Douglas, and Macfarlane[4] that platelets, antihemophilic factor (AHF), factor V, factor VII (serum prothrombin conversion accelerator, SPCA or proconvertin), and Christmas factor (plasma thromboplastin component, PTC), together with calcium were necessary for a normal thromboplastin generation test. Factor VII is now known to be unnecessary[5, 6] but the other findings of these workers have been confirmed. Several "new" clotting factors essential for "thromboplastin generation" have recently been described; these include the Hageman factor,[7] the Prower[8] and Stuart factors,[9, 10] factor X,[5] and probably plasma thromboplastin antecedent (PTA)[11] and the fourth thromboplastin component.[12] It is extremely unlikely that all these factors react together in a single high order reaction but more probable that "blood thromboplastin" is the end product of a series of intermediate reactions.[4, 13, 14] These reactions may be exceedingly complicated and Seegers[3] has stated that "the thromboplastin generation test is practically as complicated as the coagulation of whole blood and equally difficult to interpret."

There is evidence that AHF and serum interact forming an intermediate product which has been referred to as Product I.[13, 15, 16] It must be stressed that this term is used for convenience only since it has not been shown or claimed that this intermediate product is the first one formed, that only a single reaction occurs when calcium and AHF are incubated with serum or that only these factors are involved in the formation of this product. It appears from the work of Bergsagel and Hougie[13] that Product I reacts with the thromboplastic factor of platelets (platelet factor III) forming a sedimentable

From the Department of Pathology, University of North Carolina, Chapel Hill, and the Department of Clinical Pathology, University of Virginia, Charlottesville ,Va.
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*Present address: Department of Clinical Pathology, University of Virginia, Charlottesville, Va.

coagulant. The stage at which factor V is required was not established since platelets free from factor V can only be prepared from blood from a patient with factor V deficiency[17] and such blood was not available to these workers. Hörder and Sokal,[18] however, in a study of a patient with congenital factor V deficiency showed that when normal serum, AHF, and platelets prepared from the patient were preincubated with calcium for 5 minutes the subsequent addition of factor V resulted in complete activation within one minute; they concluded that factor V reacted subsequent to the reaction between AHF, serum factors, and platelets. The experiments to be described confirm this work and indicate that factor V interacts with the sedimentable complex formed by the reaction between AHF, PTC, Stuart factor, and platelets. The present study is confined to the roles of the above named factors and does not attempt to delineate the roles of PTA, Hageman factor, factor X, the Prower factor, or the fourth thromboplastin component.

METHODS AND MATERIALS

All actual clotting tests were performed in a 37° C. water bath in uncoated tubes. Silicone-coated syringes and glassware were used for collection of blood unless otherwise required by a specific method.

A patient with a congenital deficiency of factor V, a patient with Stuart factor deficiency, a severe hemophiliac (less than 3 per cent AHF), and a patient with Christmas disease were used as subjects in these experiments. The patient with the congenital deficiency of factor V (proaccelerin) has been previously reported and has been found to have 3 per cent factor V.[19] The patient with the Stuart factor deficiency has also been previously studied[8, 20] and the Stuart factor content of this patient's serum is less than 1 per cent when measured by the thromboplastin generation test.

Whole blood was collected in glass tubes and allowed to clot and stand at 28° C. for 24 hours before the serum was separated. Sera from the patient with Christmas disease and from the patient deficient in Stuart factor were treated for ½ hour with barium sulfate (50 mg. per ml.) which adsorbs Stuart factor and PTC but not factor V. The serum factors were subsequently eluted from the $BaSO_4$ by 5 per cent sodium citrate. The two preparations were then dialyzed overnight against 0.85 per cent saline and the volumes finally adjusted to the original volumes of serum. Platelets from the patient with the congenital deficiency of factor V were prepared by the technique used in the thromboplastin generation test.[21] The platelets were incubated with a variety of coagulation factors and then sedimented by centrifuging at 25,000 g for 5 minutes. The supernatant was discarded, the sediment washed once with saline and then centrifuged at 25,000 g for 5 minutes. After discarding the supernatant, the deposit was suspended in a volume of saline equal to the volume of saline in which the platelets were originally suspended. The imidazole buffer, pH 7.25, used in the reaction mixtures was prepared by the method of Mertz and Owen.[22]

RESULTS

Failure of Alumina-Treated Plasma From a Patient With Congenital Deficiency of Factor V to Form Thromboplastin.—It has been shown previously that $Al(OH)_3$-adsorbed plasma from a patient with a congenital deficiency of factor V is defective in the thromboplastin generation test but the serum is normal in respect of this test.[18] This work was confirmed (Fig. 1). The finding that substitution of normal platelets for the patient's platelets when the patient's $Al(OH)_3$-adsorbed plasma is used results in a slight but significant improvement in "thromboplastin generation"[18] was also confirmed (Fig. 1).

When normal $Al(OH)_3$-adsorbed plasma, normal serum, and normal platelets were incubated together, the "thromboplastin generated" gave the same clotting time with the patient's substrate plasma as normal plasma.

Effect of Factor V on Product I; Platelet Reaction.—In the following experiment (Table I) the barium sulfate eluates of the sera deficient in Stuart factor and PTC (diluted 1:5) were used as sources of PTC and Stuart factor, respectively, while the $Al(OH)_3$-adsorbed plasmas (diluted 1:5) from the hemophiliac and patient with factor V deficiency were used as sources of factor V and AHF, respectively. An incubation mixture of any 3 of these reagents contains all the coagulation factors present in normal serum and normal $Al(OH)_3$-adsorbed plasma with the exception of only one coagulation factor (see Table I). The 4 possible combinations of groups of 3 of the 4 reagents and a normal control mixture containing all 4 reagents were separately preincubated together in the presence of calcium for 5 minutes. Platelets from a patient with a congenital deficiency of factor V were then added to each

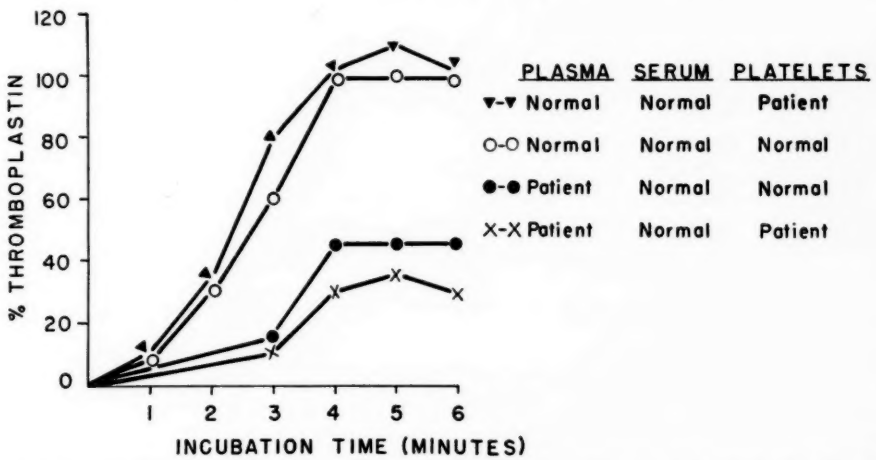

Fig. 1.—Thromboplastin generation test in a case of congenital deficiency of factor V.

of the 5 incubation mixtures and these were then further incubated for 5 minutes. The platelet material was sedimented by centrifuging, washed once and resuspended a volume of saline equal to the volume of saline in which the platelets were originally suspended. Two types of experiments were performed on the suspensions of platelet sediment. Approximately half of each suspension was diluted 1:3 with 0.85 per cent saline and the relative coagulant activity of each diluted suspension determined by adding 0.1 ml. of the suspension under test together with 0.1 ml. 0.025 M $CaCl_2$ to 0.1 ml. of a normal plasma substrate. The clotting times of the substrate plasma are shown in Table I under Column 3. In the second type of experiment, 0.2 ml. of the remaining half of each suspension of platelet sediment was incubated with 0.2 ml. of the coagulation factor which was omitted in its preparation and 0.2 ml. 0.025 M $CaCl_2$. At minute intervals after the final addition of calcium, the relative coagulant activity of each incubating mixture was determined by removing a 0.1 ml. sample and adding it together with 0.1 ml. 0.025

M $CaCl_2$ to 0.1 ml. of a normal plasma substrate. It can be seen from Table I that the platelet sediment which was prepared without factor V and had little coagulant activity showed a marked increase in activity after incubation with factor V. The maximum activity was reached after 3 minutes and was equal to that of the normal control sediment prepared using all the coagulant factors. In the case of the remaining sediments no significant increase in coagulant activity occurred when these were further incubated either with the coagulant factor omitted in their preparation or with a factor V source, viz., $Al(OH)_3$-adsorbed hemophilic plasma. Similarly no further coagulant activity resulted when the normal control platelet sediment, which was prepared with all the coagulant factors, was further incubated with factor V.

TABLE I. COMPARATIVE COAGULANT ACTIVITY OF PLATELET SEDIMENTS PREPARED FROM PLATELETS FROM CASE OF CONGENITAL FACTOR V DEFICIENCY

REAGENTS INCUBATED WITH 0.025 M $CaCl_2$, 0.6 ML. PLATELET SUSPENSION, 0.6 ML. IMIDAZOLE BUFFER	CLOTTING FACTOR ABSENT DURING THROMBOPLASTIN GENERATION	COAGULANT ACTIVITY OF PLATELET SUSPENSION			
		SUSPENDED IN SALINE		AFTER 3 MINUTES' INCUBATION WITH COAGULATION FACTOR WHICH WAS OMITTED FROM PREINCUBATION MIXTURE	
		SUBSTRATE CLOTTING TIME	PER CENT ACTIVITY	SUBSTRATE CLOTTING TIME	PER CENT ACTIVITY
		(1) sec.	(2)	(3) sec.	(4)
0.3 ml. eluate of Stuart deficient serum 0.3 ml. eluate of PTC deficient serum 0.3 ml. $Al(OH)_3$-adsorbed hemophilic plasma 0.3 ml. $Al(OH)_3$-adsorbed factor V deficient plasma	None	30	100	–	–
0.6 ml. eluate of Stuart deficient serum 0.3 ml. $Al(OH)_3$-adsorbed hemophilic plasma 0.3 ml. $Al(OH)_3$-adsorbed factor V deficient plasma	Stuart factor	79	7	71	11
0.6 ml. eluate of PTC deficient serum 0.3 ml. $Al(OH)_3$-adsorbed hemophilic plasma 0.3 ml. $Al(OH)_3$-adsorbed factor V deficient plasma	PTC	71	11	70	12
0.3 ml. eluate of Stuart deficient serum 0.3 ml. eluate of PTC deficient serum 0.6 ml. $Al(OH)_3$-adsorbed hemophilic plasma	AHF	90	2	84	5
0.3 ml. eluate of Stuart deficient serum 0.3 ml. eluate of PTC deficient serum 0.6 ml. $Al(OH)_3$-adsorbed factor V deficient plasma	Factor V	72	10	30	100

The clotting times obtained in the above experiment were converted to per cent activity from a thromboplastin dilution curve which was obtained in the following manner. The normal control suspension of platelet sediment was diluted 1:3 in saline and this dilution arbitrarily chosen to represent 100 per cent activity. Serial dilutions were made of this suspension, and the coagulant activity of 0.1 ml. of each dilution determined. Thus, 0.1 ml. of a dilution was added simultaneously with 0.1 ml. M $CaCl_2$ to 0.1 ml. normal plasma and the clotting time of the mixture determined. A dilution curve, relating clotting time of the mixture to the strength of each dilution added to the clotting mixture, was then prepared.

Comparison of One-Stage "Prothrombin Times" of Normal and Factor V Deficient Plasma Using Product I-Treated Platelets Prepared With and Without Factor V as Thromboplastin Source.—Product I-treated platelet sediments were prepared by preincubating for 5 minutes, 0.2 ml. normal serum diluted 1:10 (source of both PTC and Stuart factor), 0.2 ml. imidazole buffer and 0.2 ml. 0.025 M $CaCl_2$ with 0.2 ml. of 1:5 dilution of each of the following: (a) normal $Al(OH)_3$-adsorbed plasma and (b) $Al(OH)_3$-adsorbed plasma from patient deficient in factor V. To each of the 2 mixtures was then added 0.8 ml. of a platelet suspension from the patient with factor V deficiency and incubation carried out for a further 5 minutes. The subsequent technique was as described in the section on methods. The 2 final suspensions of platelet sediments were diluted 1:3 in .85 per cent saline. The one-stage "prothrombin times" of normal plasma and plasma from the patient with congenital deficiency of factor V were then determined using the sediments prepared with and without factor V as the source of thromboplastin.

TABLE II. MODIFIED ONE-STAGE "PROTHROMBIN TIMES" OF NORMAL AND FACTOR V DEFICIENT PLASMAS USING PLATELET SEDIMENTS PREPARED FROM PLATELETS FROM CASE OF CONGENITAL FACTOR V DEFICIENCY

NORMAL SERUM, PATIENT'S PLATELETS, IMIDAZOLE BUFFER, $CaCl_2$ INCUBATED WITH	ONE-STAGE "PROTHROMBIN TIMES" (SEC.) USING "PLATELET SEDIMENTS" AS SOURCE OF THROMBOPLASTIN	
	NORMAL PLASMA SEC.	FACTOR V DEFICIENT PLASMA SEC.
Normal $Al(OH)_3$-adsorbed plasma	24	22
Factor V-deficient $Al(OH)_3$-adsorbed plasma	47	95

The results of such an experiment are shown in Table II. It can be seen that the "prothrombin time" of the factor V deficient plasma was normal when the platelet sediment prepared with factor V was used as the source of thromboplastin and that the "prothrombin times" of both the normal and factor V deficient plasma were prolonged when the platelet sediment prepared without factor V was used as the thromboplastin source.

DISCUSSION

The results confirm earlier work[13] that a sedimentable coagulant forms when all the factors essential for "thromboplastin generation" are incubated

together. The present work also shows that if any one of the following, AHF, PTC, Stuart factor, or factor V is omitted from the preparation of the sedimentable coagulant, it will have comparatively little activity. Subsequent incubation of the defective sediment with the coagulation factor originally omitted does not result in further increase of coagulant activity, except in the case of factor V. It appears, therefore, that factor V acts subsequent to PTC, Stuart factor, platelets, and AHF on the sedimentable complex formed by these factors. While the possibility that factor V may act earlier under more physiologic conditions of clotting is not excluded by either this work or the previous work of Hörder and Sokal,[18] it is rendered unlikely by indirect evidence. Thus, it has been recently shown by Douglas[23] that when whole blood from a patient with hemophilia, Christmas disease (congenital PTC deficiency), or thrombocytopenia is allowed to clot in a glass tube there is a failure to utilize factor V. These findings suggest that factor V enters the reactions involved in blood clotting at a later stage than AHF, Christmas factor, and platelets. If the factor V had been involved in the earlier stages of thromboplastin formation then its utilization in one or more of these conditions would have been anticipated.

The findings can all be fitted into the following scheme which is similar to that suggested by Macfarlane.[16]

(1) PTC + AHF + Stuart factor + Calcium → Intermediate Product I
(2) Intermediate Product I + Platelets → Intermediate Product II (sedimentable)
(3) Intermediate Product II + factor V → Blood Thromboplastin or final prothrombin converting substance (sedimentable)

Such a hypothesis is necessarily incomplete. It does not take into account the Hageman factor, PTA, factor X, and other factors thought to be important in "thromboplastin generation," nor does it consider inhibitory systems. The scheme, however, agrees in part with the view of Owren[24] that antihemophilic "A" and "B" factors (AHF and PTC) and platelets react to form a product which then reacts with proconvertin (factor VII) to form "convertin" (probably the Product II of the above scheme). "Convertin" then reacts with "accelerin" (derived from factor V) to form blood thromboplastin or "prothrombinase," the final prothrombin-converting substance. Since it is now known that factor VII (proconvertin) is not essential for "blood thromboplastin," it is probable that Owren was considering the Stuart factor rather than factor VII (proconvertin) since these 2 factors have many properties in common and the "specific" proconvertin assay technique of Owren and Aas[25] measures not only proconvertin but Stuart factor and perhaps other factors as well.[8] Owren's view that proaccelerin (factor V) is converted to accelerin by the action of thrombin is generally accepted.[26, 27] It has, however, been previously shown that washed sediments, prepared by the technique used in the present work, do not contain demonstrable thrombin.[13] The thrombin necessary for this

transformation of factor V must therefore be derived from traces of prothrombin present in the factor V source ($Al[OH]_3$-adsorbed hemophilic plasma), or be incorporated into the sediments as previously suggested by Spaet and Garner[28] and, therefore, not demonstrable by the usual techniques.

CONCLUSIONS

Experimental evidence is presented indicating that factor V is not required in the formation of Product I thereby differing in this respect from PTC, Stuart factor, and AHF, which are necessary for the formation of this product. Product I appears to react with the thromboplastin factor of platelets and this in turn reacts with factor V.

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VITAMIN B_{12} BINDING CAPACITY OF NORMAL AND LEUKEMIC SERA

G. RACCUGLIA, M.D.,* AND M. S. SACKS, M.D.**
BALTIMORE, MD.

INTRODUCTION

The possibility of assaying vitamin B_{12} by the use of the green alga *Euglena gracilis* var. *bacillaris* was first demonstrated by Hutner and his associates.[1] Ross[2] described in particular a technique by means of which it is possible to determine the concentration of the vitamin in body fluids. Beard, Pitney, and Sanneman[3] and Beard et al.[4] described an abnormally high concentration of vitamin B_{12} in the patients suffering from acute or chronic granulocytic leukemia and acute monocytic leukemia. Patients suffering from acute or chronic lymphocytic leukemia were described as having normal levels of the substance. These findings were confirmed by a number of investigators.[5, 6] Pitney, Beard, and Van Loon[7] demonstrated also that the sera of patients suffering from chronic granulocytic leukemia can bind more vitamin B_{12} than normal serum. This finding was confirmed by Lear and his associates.[8] The purpose of this paper is to present a method useful for the measurement of the vitamin B_{12} binding capacity of serum and the results obtained with normal and pathological sera.

MATERIALS AND METHODS

The assays of vitamin B_{12} were carried out with the method of Ross[2] with only minor modifications. With this technique, it is possible to differentiate a "bound" form of the vitamin, which becomes available to the microorganism after heating the sera to 100° C., from a "free" form, which is available to the microorganism even before heating. In normal sera, the latter form is usually found in very small amounts. If vitamin B_{12} is added to a normal serum and this mixture is inoculated with *E. gracilis* without previous heating, the growth of the microorganism will be less than it would be if the same amounts of vitamin B_{12} were added to distilled water instead of serum. The interpretation of this phenomenon is that the vitamin B_{12} added to the serum becomes linked with a globulin component,[7] and in this form the substance does not support the growth of *E. gracilis*. Our method for the measurement of the binding capacity of sera was developed on the basis of this hypothesis. Serial dilutions of sera from 1:1 (pure serum) to 1:64 were prepared using sterile distilled water as diluting fluid. To 1 milliliter of each of these dilutions, 1 milliliter of a solution of crystalline vitamin B_{12} containing 800 $\mu\mu$g of substance per milliliter was added. No incubation of the mixtures was done at this point, but the tubes were left at room temperature during the execution of the other steps of the technique. The concentration of "free" vitamin B_{12} in each of these dilutions was assayed. To obtain it, 0.10 milliliter of each mixture was added to 1.9 milliliters of water and 2.0

From the University of Maryland School of Medicine.
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Received for publication Dec. 20, 1956.
*Instructor of Medicine. Present address: Simpson Memorial Institute, University of Michigan, Ann Arbor, Mich.
** Professor of Clinical Medicine.

milliliters of double strength medium. The tubes were then incubated for 15 minutes at 56° C. This degree of heating does not liberate any of the bound vitamin B_{12} naturally present in normal sera; its purpose is to decrease the possibility of growth of contaminant fungi in the material.

If the concentration of naturally present "free" vitamin B_{12} in the serum, at the dilutions used for the assays, is zero, the vitamin B_{12} recovered should represent the amount of added substance that the serum did not bind. The amount recovered should be greater at the higher dilutions of sera and should be 100 per cent where the vitamin is added to water instead of serum. The free vitamin B_{12} concentration at these dilutions is actually zero in practically all normal sera, but it is sometimes increased in abnormal sera. In this case, the pre-existing amount of vitamin B_{12} should be subtracted from the value found after the addition of crystalline vitamin B_{12}, and the difference will indicate the amount of added substance which actually remained unbound. To accomplish this, a second set of tubes was prepared, containing the sera treated in the way described above, except that to the sera was added distilled water instead of the solution containing vitamin B_{12}. When vitamin B_{12} could be detected in this set of tubes, the values were subtracted from the corresponding ones of the first set.

Plotting the amounts of vitamin B_{12} recovered against the dilutions of sera (Fig. 1), it is possible to obtain a curve from which the binding capacity of a given serum can be estimated. In Fig. 1, it can be noted that the amounts of vitamin B_{12} recovered were sometimes greater than the amounts added; these insignificant differences are within the limits of error for the method employed. All the assays were carried out in triplicate.

RESULTS

Determinations of total and free vitamin B_{12} serum levels were caried out in 180 normal individuals, 59 cases of miscellaneous diseases, 12 cases of pernicious anemia, and 50 cases of leukemia. The values obtained are shown in

TABLE I. SERUM VITAMIN B_{12} IN NORMAL AND PATHOLOGICAL SERA ($\mu\mu$G PER MILLILITER)

NO. OF PATIENTS	DIAGNOSIS	RANGE		AVERAGE	
		TOTAL	FREE	TOTAL	FREE
180	Normal	144-1,080	0-104	387*	25
59	Miscellaneous diseases	22-2,200	0-272	672	38
12	Pernicious anemia	0-50	0	34	0
26	Chronic granulocytic leukemia	2,368-16,600	0-216	7,701	88
9	Acute granulocytic leukemia	300-3,580	0-204	988	71
6	Chronic lymphocytic leukemia	140-760	0-328	416	111
7	Acute lymphocytic leukemia	234-860	42-304	497	145
2	Monocytic leukemia	1,376-1,520	0-872		

*Standard deviation ± 140.

Table I. Twenty-four of the normal sera were studied with the method described above in order to determine their binding capacity. The original levels of total vitamin B_{12} in these sera ranged from 218 to 1,024 $\mu\mu$g per milliliter and those of the "free" vitamin B_{12} from 0 to 104. When these normal sera were diluted, only a small percentage of the added vitamin B_{12} became bound. At the lower dilutions of sera, the binding capacity was higher, but some of the added vitamin was always recoverable. In most of the experiments, the vitamin bound was between 50 per cent and 80 per cent (between 400 and 650 $\mu\mu$g per milliliter). Fig. 1 shows the average

binding capacity curve and the standard deviations obtained with normal sera. The same technique was used for the study of our 26 cases of chronic granulocytic leukemia, before treatment. Fig. 2 shows the average curve and standard deviations of the results. The binding capacity was always very high, and the free vitamin often completely disappeared even when there was less than 10 per cent of the serum present. Thirteen of these patients were followed during the course of their disease with repeated assays of vitamin B_{12}. Twelve of them were treated with Myleran, 6 mg. per day. Three patients received a course of x-ray therapy when they became resistant to Myleran therapy. One received only x-ray therapy. The total vitamin content of the serum of these patients decreased consistently with the reduction in the white blood count, and

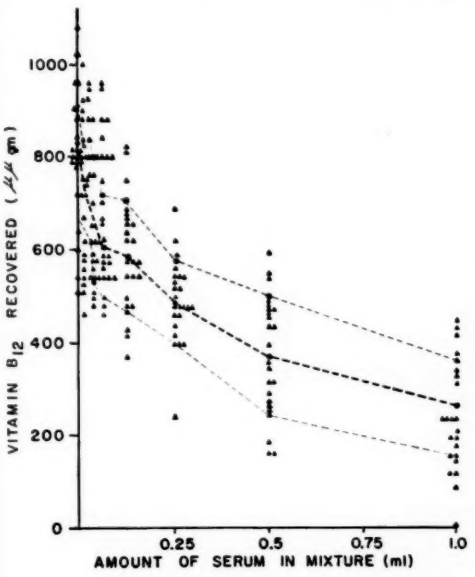


Fig. 1.

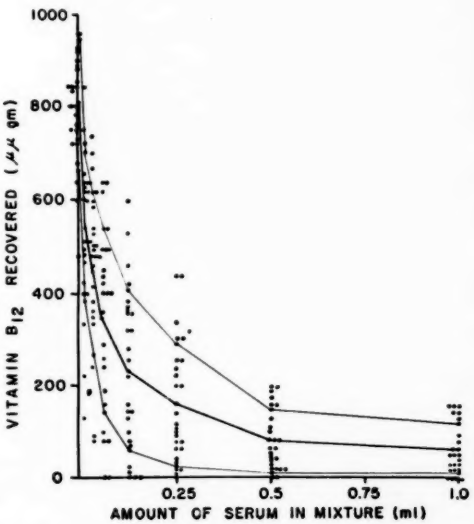


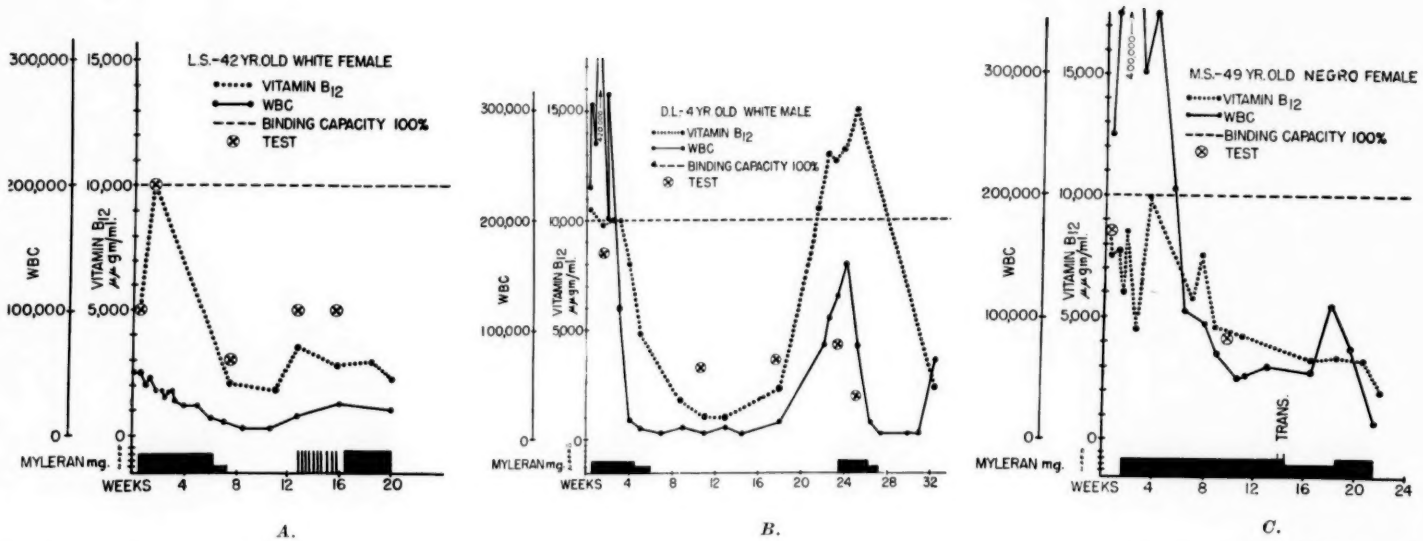
Fig. 2.

Fig. 1.—Vitamin B_{12} binding capacity of 24 normal sera (average curve and standard deviations).

In Figs. 1, 2, 4, 5, and 6, the amount of vitamin B_{12} bound is represented by the difference between the amount of vitamin B_{12} added and the amount of vitamin B_{12} recovered.

Fig. 2.—Vitamin B_{12} binding capacity of 26 sera of patients with chronic granulocytic leukemia. (Average curve and standard deviations.)

increased again when the number of leukocytes rose. In Fig. 3 (*A*, *B*, and *C*), the binding capacities of the sera are represented by one value only. This is the amount of vitamin B_{12} bound by the sera at a dilution of 1:8. The choice of the serum binding capacity at this dilution was arbitrary. Fig. 4 shows the average curve and standard deviations of the results obtained in these 13 patients when, after a course of Myleran therapy, their white blood count reached normal values. In this sera, the total vitamin B_{12} ranged from 350 to 1,860 $\mu\mu$g per milliliter, the "free" from 0 to 216. The results obtained in the untreated chronic granulocytic leukemia are shown in the same figure for comparison. Five cases of acute lymphocytic leukemia and 6 of chronic lymphocytic leukemia



A. *B.* *C.*

Fig. 3.—*A, B, C,* Number of leukocytes, serum levels of vitamin B_{12}, and serum vitamin B_{12} binding capacity in three patients with chronic granulocytic leukemia under treatment with Myleran.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 4.—Vitamin B_{12} binding capacity of 13 sera of patients with chronic granulocytic leukemia after effective treatment (▲--▲--▲) in comparison with vitamin B_{12} binding capacity of 25 sera of patients with chronic granulocytic leukemia before treatment (●—●) (Average curves and standard deviations.)

Fig. 5.—Vitamin B_{12} binding capacity of 11 sera of patients with lymphocytic leukemia. (Average curves and standard deviations.)

Fig. 6.—Average curves of vitamin B_{12} binding capacity of serum obtained from normal individuals (▲--▲--▲), patients with chronic granulocytic leukemia (●—●), and lymphocytic leukemia (✕••✕••✕).

in whom the serum content of vitamin B_{12} varied from 140 to 860 $\mu\mu$g per milliliter for the total form and from 0 to 328 for the "free" form, were also studied with the method described above. The average curve and standard deviations of the results are shown in Fig. 5.

In addition, the serum binding capacity was studied in 3 cases of acute granulocytic leukemia, 3 cases of monocytic leukemia, and 6 cases of untreated pernicious anemia. The curves obtained with the serum from these patients were all within normal limits and are not reported here.

In Fig. 6, the average curves obtained in normal, lymphocytic leukemia and chronic granulocytic leukemia sera are shown together for comparison.

DISCUSSION

The simple vitamin B_{12} determinations in our series of patients showed results comparable with those obtained by others. Namely, there was an increased amount of total vitamin B_{12} in the serum of patients suffering from chronic granulocytic leukemia, while in serum of patients suffering from acute and chronic lymphocytic leukemias, the values of total vitamin B_{12} were within normal limits.

When pure vitamin B_{12} was added to the different types of sera, a striking difference in the recovery of the substance was observed. The vitamin added to normal sera never became entirely unavailable to the microorganism, while the substance added to sera obtained from patients suffering from chronic granulocytic leukemia was often completely undetectable by assays even at the very high dilutions of sera. The sera of patients suffering from lymphocytic leukemia, acute or chronic, bound less vitamin than chronic granulocytic leukemia and even less than normal serum. The levels of "free" vitamin B_{12} in acute and chronic lymphocytic leukemia were elevated in comparison with those of normal and of chronic granulocytic leukemia (Table I).

The high values of serum vitamin B_{12} in chronic granulocytic leukemia decreased progressively as the patient responded favorably, with lowering of the leukocyte levels, to treatment with either Myleran or x-ray therapy. When the number of white blood cells fell to normal values, the binding capacity of the sera also became normal. In Fig. 3, the index of binding capacity is arbitrarily represented as the amount of vitamin B_{12} recovered when 1 ml. of a solution containing 800 $\mu\mu$g of the substance was added to 1 ml. of serum diluted 1:8. It can be seen that the amounts of vitamin B_{12} bound were higher when the serum levels of the vitamin were high, namely, during relapse when the leukocyte count was more elevated. Comparison between the binding capacity curves obtained in patients before and after treatment shows a striking difference (Fig. 4). Reduction of the total serum vitamin B_{12} and of the binding capacity was not observed regularly in patients whose disease was advanced and becoming clinically refractory to any treatment even though there was a fall in the leukocyte count. This is in agreement with the finding of Mollin and Ross.[6]

These findings seem to support the hypothesis that the high levels of vitamin B_{12} in chronic granulocytic leukemia are in some way connected with the elevated

number of leukocytes. The decreased serum vitamin B_{12} binding capacity and the increase of the "free" form in chronic lymphocytic leukemia suggest a relationship between the absence of granulocytes and diminished avidity of serum for vitamin B_{12}. The hypervitaminosis observed in cases of nonleukemic leukocytosis[4, 6] is also in agreement with this concept. Were such the case, the vitamin should originate from the granulocytes or the substance should become bound, after intestinal absorption, to a protein liberated or produced by the leukocytes. This explanation seems to be the simplest one and, if such a hypothesis is excluded, the interpretation of the phenomenon of hypervitaminosis is more difficult.

Nevertheless, many other findings cannot be explained by these hypotheses. Beard and his associates[3] observed a correlation between leukocytosis and levels of vitamin B_{12} in the serum of chronic granulocytic leukemia patients but this correlation was not evident in cases of acute leukemias.[4] Harris[9] showed that normal leukocytes contain significant amounts of vitamin B_{12} but Thomas and Anderson[10] showed that the amount of vitamin B_{12} in the leukocytes of patients suffering from leukemia is not increased. Molin and Ross[6] were unable to demonstrate that the leukocytes were the source of vitamin B_{12}. They showed that the leukocytes could release material capable of binding vitamin B_{12}. Thomas and Anderson,[10] however, demonstrated that leukemic leukocytes do not differ from normal leukocytes in their vitamin B_{12} binding capacity. If the increased serum level and the increased binding capacity are connected with the presence of graulocytes, one would expect a decrease of the substance and of the binding capacity in cases of aplastic anemia or agranulocytosis. Neither of these phenomena was, however, observed by Beard et al.,[3] Mollin and Ross,[6] or by ourselves.

In pernicious anemia, the curves of the binding capacity were normal in spite of the lower vitamin B_{12} content of the serum and the leukopenia. We observed, moreover, that while the levels of vitamin B_{12} vary in general with the fluctuations of the leukocyte count in *a single patient*, the vitamin B_{12} content is not necessarily directly related to the number of granulocytes in circulation. In other words, if two subjects have a leukocyte count of 40,000, in one there may be a serum level of vitamin B_{12} of 2,000 $\mu\mu$g per milliliter and in the other of 10,000 $\mu\mu$g per milliliter. We have not observed serum levels higher than 20,000 $\mu\mu$g per milliliter even with extreme leukocytosis.

Falling serum vitamin B_{12} levels during treatment of chronic granulocytic leukemia seems to parallel the decrease in leukocytes. Only occasionally a questionable transient rise of the serum vitamin B_{12} could be observed after initiation of effective treatment followed by the fall of the values. This phenomenon would perhaps be of greater magnitude if the vitamin B_{12} originated from the leukocytes. Perhaps the most convincing argument against the granulocytes as the source of serum vitamin B_{12} is the fact that hypervitaminosis is not limited to chronic granulocytic leukemia but is found also in cases of cirrhosis of the

liver,[8, 11] infectious hepatitis,[11] and polycythemia vera[6] in which diseases elevation of the number of leukocytes is not an obligatory feature. The vitamin B_{12} binding capacity of serum in these diseases was not measured.

Rachmilewitz, Aronovitch, and Grossowicz[11] postulated that the increase of serum vitamin B_{12} in liver diseases is due to the release of the stored vitamin from damaged liver cells. While this is possible, the excess vitamin would probably be rapidly excreted unless there were greater retention. Such increased retention has been shown to be present in chronic granulocytic leukemia[7] and is presumably due to enhanced binding capacity of the plasma. Another interpretation of this phenomenon is that in certain diseases and especially in chronic granulocytic leukemia, the plasma contains an abnormal protein or an increased amount of a normal protein which binds vitamin B_{12}. By means of electrophoresis, it is possible to locate the zone where vitamin B_{12} is bound. This is found at the level of the alpha-1 and alpha-2 globulins, both in normal and in leukemic sera.[7] No qualitative differences could be demonstrated by this technique.

No differences in protein content can be detected between normal and leukemic sera by the usual chemical means. Nevertheless, the possibility of minimal quantitative differences cannot be excluded since the amounts of vitamin assayed are extremely small and the method used for the measurement of serum protein is, of course, much less sensitive than the microbiological method used for vitamin assays.[6] An increase of the globulins has been demonstrated in the serum of chronic granulocytic leukemia[12-14] with more refined techniques.

In conclusion, it seems that the phenomenon of hypervitaminosis B_{12} in chronic granulocytic leukemia can best be explained by assuming as is probably the case, that the cyanocobalamin is absorbed in normal amounts, but in the presence of increased quantities of a binding protein, apparently an alpha globulin, the vitamin remains in the plasma until high levels are attained. The source of this binding material is unknown. The hypothesis that the binding substance originates from the leukocytes deserves further investigation. It is possible that the method for the measurement of binding capacity described in the present paper can provide an indirect estimation of this vitamin B_{12} binding substance.

SUMMARY

A method for the measurement of binding capacity of serum for vitamin B_{12} is described. With this method, sera from normal subjects and patients suffering from chronic granulocytic leukemia, lymphocytic leukemia (acute or chronic), and various other conditions were studied. The serum of chronic granulocytic leukemia patients showed an increase and that of lymphocytic leukemia patients a decrease of the binding capacity for vitamin B_{12}.

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THE ARGINASE ACTIVITY OF ERYTHROCYTES AND LEUKOCYTES WITH PARTICULAR REFERENCE TO PERNICIOUS ANEMIA AND THALASSEMIA MAJOR

JOHN REYNOLDS, M.D., JAMES H. FOLLETTE, M.D., AND
WILLIAM N. VALENTINE, M.D.
LOS ANGELES, CALIF.

ARGINASE has long been known to be present in various human tissues. This enzyme was first described in 1904,[1] and subsequently it has been identified in several human tissues including liver, breast, kidney, testes, salivary glands, epidermis, and erythrocytes. Its activity in liver has repeatedly been shown to be greater than that of other tissues and many times that of blood. Krebs and Henseleit[2] established the importance of hepatic arginase in the cycle by which amino nitrogen is converted into urea during the catabolism of amino acids. The physiologic significance of arginase in other tissues has never been clearly defined. Arginase has long been considered one of the most specific of enzymes. It has been found in both the cytoplasm and nuclei of cells, but as yet there is no satisfactory histochemical technique for its measurement. No attempt will be made to further summarize the existing knowledge about this enzyme since excellent reviews are available.[3]

Several investigators have concerned themselves with the arginase activity of human blood. Weil and Russell[4] were among the first to demonstrate its presence in human erythrocytes. Covolo and West[5] concluded that red cell arginase activity varied with each individual. The results of Clark and Beck[6] suggested that there was no significant variation in the activity of this enzyme with aging or pregnancy. Kochakian and his associates[7] measured the activity of erythrocyte arginase in various diseases and were the first to point out an increased value in pernicious anemia, either untreated or in relapse. They further demonstrated that the erythrocyte arginase activity decreased progressively as these patients showed hematologic improvement. They also suggested from their data that a diurnal variation in enzyme activity exists, that there is no relation of activity to meals, and that levels for a given individual were not constant when serial assays were performed over a year.

Although the existence of arginase has been denied in human leukocytes,[7] the purpose of the present study was to assay human leukocytes and erythrocytes for arginase in the normal as well as the abnormal individual, applying newer methods for the separation of formed element of blood.

From the Department of Medicine, School of Medicine, University of California Medical Center, and the Veterans Administration Center, Los Angeles, Calif.

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METHODS

Separation of erythrocytes from leukocytes was accomplished in the manner previously described.[8-10] A 4 per cent solution of bovine fibrinogen (Sigma) was found to provide optimal yields. The entire procedure was carried out in a cold room at environmental temperature of 4° C. After sedimentation of the red cells, the leukocyte-rich supernatant plasma was aspirated. Leukocytes were separated from the platelets and plasma by centrifugation at 1,000 r.p.m. for 10 minutes. Thereafter, the leukocytes were washed twice with normal saline. The preincubation suspension of white cells was adjusted so as to give a final cell concentration of 45,000 to 50,000 per cubic millimeter. This final suspension was then counted in quadruplicate, using Bureau of Standards' certified red cell pipettes. An erythrocyte contamination of about 50,000 cells per cubic millimeter was considered insignificant in so far as no detectable arginase activity could be demonstrated on repeated arginase determination in this range of pure erythrocyte concentration.

The erythrocytes were processed in a slightly different way. After aspiration of the leukocytes, the remaining erythrocyte-plasma suspension was washed twice with saline at centrifugation speeds of 1,500 r.p.m. Platelet contamination was minimal. Moreover, assay of highly concentrated platelet suspensions for arginase had previously given negative results.[11] The erythrocyte suspension in saline was then adjusted by dilution to provide a concentration of 1.0 to 1.5 million cells per cubic millimeter so that final colorimeter readings would fall within a range of optical densities conforming to Beer's law. After quadruplicate cell counts had been taken, this suspension was centrifuged at 2,500 r.p.m. for 10 minutes, the supernatant saline was aspirated and discarded, and the original volume was restored with glass-distilled water. Neither homogenization of cells in distilled water nor addition of saponin appeared to increase the enzyme yield from white cells. Consequently the leukocytes were intact in saline suspension at the time of addition to the substrate. By way of contrast, enzyme activity of erythrocytes was negligible unless these cells were hemolyzed in distilled water prior to incubation. Addition of saponin failed to further increase erythrocyte arginase activity.

Arginase activity was determined by a modification of the methods of Kochakian[7] and Kingsley.[12] The following reagents were used:

1. L-arginine monohydrochloride, 0.047 M (1 per cent) solution in glass-distilled water, pH adjusted to 9.9
2. Diethylbarbiturate buffer, pH adjusted to 9.9
3. Cobaltous chloride, 0.0015 M solution in glass-distilled water
4. Hydrochloric acid, 0.3 N
5. Phosphate buffer (3 parts 0.2 M KH_2PO_4 to 7 parts 0.2 M Na_2HPO_4 solution). The pH must be adjusted to a point (ca. 12.0) so that addition of 1.0 ml. of this buffer to the arginase digest will lower the pH of the resultant mixture to 7.0
6. Urease solution (1.0 Gm. of urease, 1.5 Gm. of Permutit, and 15 Gm. of sodium chloride are brought to a volume of 50 ml. with glass-distilled water, mixed by shaker for 30 minutes, allowed to stand at room temperature for 2 hours, filtered through Whatman No. 42 paper and adjusted to pH of 7.0)
7. Stock standard urea solution (400 mcg. of urea N per milliliter)
8. Acid cadmium sulfate solution (5.77 Gm. of $3CdSO_4.8\ H_2O$ and 28.2 ml. of 1 N.H_2SO_4 are brought to a volume of one liter with distilled water)
9. Sodium hydroxide 1.1 N
10. Nessler's Reagent

For each determination 2 unknowns, a blank and a standard were run. Each unknown incubation mixture consisted of 2.0 ml. of arginine solution, 1.0 ml. of barbiturate buffer, 0.25 ml. of cobaltous chloride solution, and 0.75 ml. of glass-distilled water. After equilibration in a water bath at 38° C., 1.0 ml. of cell suspension was added to each unknown. The blank contained the same constituents except for the addition of 1.0 ml. of 0.3 N HCl.

Upon addition of the cells the blank tube was placed in a boiling water bath for 5 minutes in order to arrest all enzyme activity. The standard solution differed from the blank only by the substitution of 0.75 ml. of stock urea standard for glass-distilled water.

After addition of the white cells or the laked red cells, each solution was thoroughly mixed and incubated in a 38° C. water bath for 6 hours. Mixing at half-hour intervals was necessary to prevent settling of the protein coagulum. Each test tube was covered with a spherical marble to minimize diminution of volume by vaporization. At the end of 6 hours the arginase was inactivated by adding 1.0 ml. of 0.3 N HCl, mixing, and immersing the unknown test tubes in a boiling water bath for 5 minutes. Sufficient time was allowed for cooling before the addition of 1.0 ml. of phosphate buffer. A heavy brown precipitate was then formed by thoroughly mixing and was discarded after centrifugation at 2,500 r.p.m. for 10 minutes. The resulting supernatant could be saved for at least 3 days in a cold room or refrigerator before exposure to the action of urease.

In order to measure the urea so formed, 3 drops of urease solution were mixed with a 0.5 ml. aliquot of each unknown, blank, and standard supernatant, preheated in a water bath at 38° C. After frequent shaking throughout, this incubation was terminated at the end of 30 minutes by addition of 8.5 ml. of acid cadmium sulfate solution. Calibrated syringe pipettes were found useful for this purpose. After one minute 1.0 ml. of 1.1 N NaOH was added. The resultant mixture was shaken thoroughly to enhance flocculation and then allowed to stand 10 minutes before centrifugation at 2,500 r.p.m. for 10 minutes.

Final determinations were made in a Beckman Model-B spectrophotometer, using a blue lamp and wave length of 540 mμ. A 2.0 ml. aliquot of each clear supernatant was pipetted into spectrophotometer tubes. Nine milliliters of distilled water was then added by calibrated syringe pipette to each tube and carefully mixed. One milliliter of Nessler's reagent was added, and the optical density of the resultant amber solution read within one minute to avoid spurious readings caused by fine colloidal turbidity.

The values for arginase activity were expressed as milligrams of urea nitrogen per 10^{10} W.B.C. or per 10^{11} R.B.C. liberated in one hour from arginine substrate at 37° C. The figure for erythrocytes was chosen in order to avoid decimals below unity.

RESULTS

The erythrocyte and leukocyte unit cell arginase values are presented in the form of scattergrams (Figs. 1 and 3) in order to reflect the differences among several disease groups.

Erythrocytes.—Normal subjects: These were comprised of patients hospitalized for elective surgical conditions such as herniae and for chronic nonhematologic disorders such as arteriosclerotic brain disease. These patients were studied before surgery and all were in apparent good health apart from some nonsystemic disease. All patients were adults, and the majority were men. No attempt was made to analyze the results with regard to sex or age. Subjects were considered "normal" only if their hemoglobin, white blood count, and differential white blood count were within normal limits. Twenty-four such subjects were studied and values ranging from 0 to 46.2 mg. per 10^{11} R.B.C. were observed. The mean value was 21.0. In several instances repeat determinations were found to agree closely with the original value. All but 3 of the values fell within a range of 9.0 to 36.0, although it is quite probable that an increased number of normal subjects would show a somewhat greater spread. The normal mean erythrocyte arginase activity per unit cell was found to be about one-fiftieth that of normal leukocytes.

Pernicious and other nutritional macrocytic anemias: Sixteen patients with megaloblastic anemia (Table I) were studied while in hematologic relapse

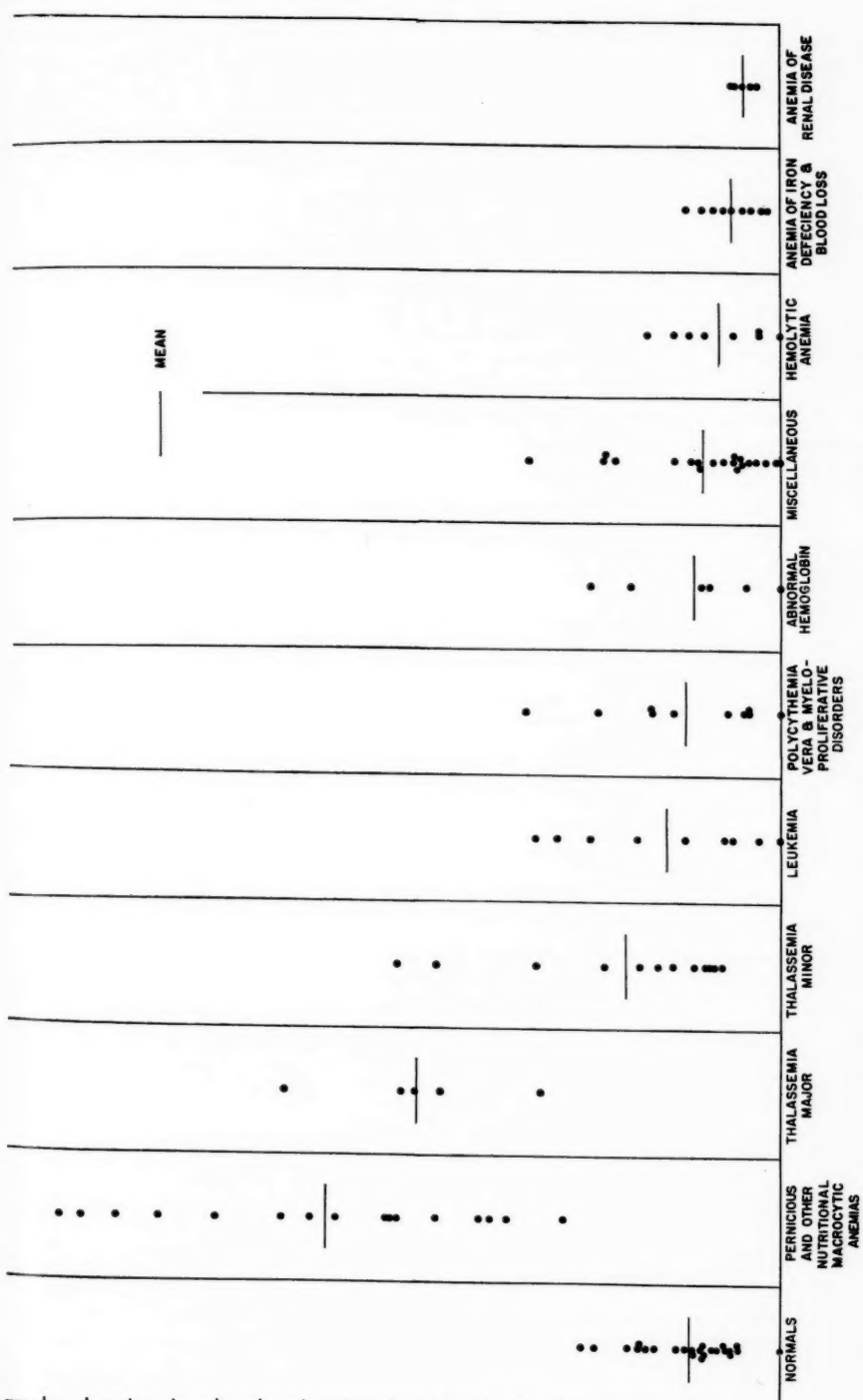

Fig. 1.—Scattergram of arginase activity of erythrocytes obtained from normal human subjects and subjects with various disease states. Each dot represents a different patient. Fourteen patients had typical Addisonian pernicious anemia in relapse, while the other two in the same column had a vitamin B_{12} responive megaloblastic anemia associated with regional enteritis. Values for arginase activity are expressed as milligrams of urea nitrogen liberated per 10^{11} erythrocytes.

TABLE I. ERYTHROCYTE ARGINASE IN PERNICIOUS AND OTHER NUTRITIONAL MACROCYTIC ANEMIAS

PATIENT	DIAGNOSIS AND REMARKS	HEMOGLOBIN* (GM. PER 100 ML.)	R.B.C. ARGINASE* (MG. PER 10^{11} R.B.C.)
C. P.	Pernicious anemia in partial relapse with reticulocytosis on vitamin B_{12} therapy	9.7	50.2
J. M.	Pernicious anemia with positive therapeutic test to I.M. vitamin B_{12}	11.1	63.6
B. C.	Pernicious anemia. Megaloblastic anemia with suboptimal reticulocytosis following vitamin B_{12} because of overwhelming decubiti and bronchopneumonia	7.3	67.6
E. V.	Pernicious anemia and subacute combined system disease. 0.75% urinary excretion of CO^{60} B_{12} (Schilling Test)	7.0	70
T. W.	Pernicious anemia. See figure for response to oral folic acid	5.7	79.8
I. A.	Pernicious anemia. Megaloblastic anemia; 0% urinary excretion of CO^{60} B_{12} (Schilling Test). M.C.V. 134	11.6	89.4
G. H.	Pernicious anemia. Megaloblastic marrow. Arginase normal after third week of vitamin B_{12}	5.9	90.6
L. R.	Pernicious anemia with subacute combined degeneration; megaloblastic bone marrow. Appropriate reticulocyte and hemoglobin response to vitamin B_{12}	6.8	91.6
	Mean arginase and mean hemoglobin	7.4	105.4
C. K.	Pernicious anemia. Achlorhydria; megaloblastic marrow. Age 47	8.6	103
N. D.	Pernicious anemia in relapse (see Fig. 2 for response to I.M. vitamin B_{12})	6.9	108.6
L. M.	Untreated pernicious anemia; megaloblastic bone marrow; 44% reticulocytosis on vitamin B_{12}. Histamine achlorhydria	4.8	115.4
F. Mc.	Inadequately treated pernicious anemia. 95% fecal excretion of oral CO^{60} B_{12}	9.8	130.8
P. H.	Pernicious anemia. Megaloblastic marrow	8.0	144
J. B.	Longstanding regional enteritis, complicated by small bowel resections, fistulae, and abscesses. Macrocytic anemia responsive to parenteral vitamin B_{12}. Megaloblastic bone marrow	7.3	153.8
H. C.	Pernicious anemia following gastric resection 9 years earlier	3.8	161.7
M. S.	Regional enteritis. Free acid present. Dramatic reticulocytosis and clinical improvement on I.M. vitamin B_{12}. P.C.V. of 8%	3.0	167

*Values obtained either before or at the onset of specific hematinic therapy.

or at the time of initiation of appropriate treatment. Fourteen of these were considered to exhibit typical Addisonian pernicious anemia by virtue of histamine anacidity, a bone marrow showing typical megaloblasts, a distinct reticulocyte response to either B_{12} or folic acid therapy, and in several instances characteristic fecal or urinary excretion patterns of radioactive cobalt, as described in the methods of Heinle[13] and Schilling.[14] Two patients suffered from regional enteritis complicated by multiple surgical procedures. Both showed free hydrochloric acid at the time of gastric analysis, and both responded well hematologically to parenteral administration of vitamin B_{12}.

The values for erythrocyte arginase of these 16 patients ranged from 50.2 to 167 with a mean of 105.4. As can be seen from the scattergram, all values are well in excess of that of the highest normal subject. The mean of the untreated megaloblastic anemias is more than 5 times that of normal subjects.

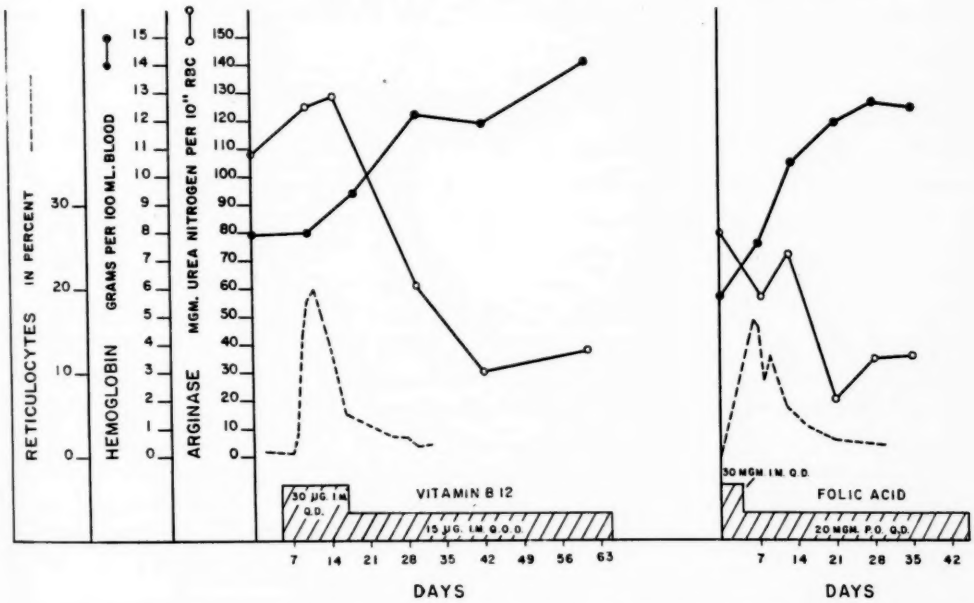

Fig. 2.—Progressive lowering of erythrocyte arginase activity in two different patients with pernicious anemia. One received only vitamin B_{12}, and the other received only folic acid during the period of study. Each point represents a separate arginase, hemoglobin, and reticulocyte determination.

Eight of these patients were followed serially over periods up to 90 days as they were receiving treatment. One patient, who showed no signs of subacute combined system disease, was purposely placed on folic acid therapy for a period of one month in an attempt to compare his arginase response to that of patients receiving vitamin B_{12} therapeutically. He developed a typical reticulocyte and hemoglobin response while on folic acid (Fig. 2) as well as a progressive diminution of erythrocyte arginase activity. In all instances of serial determinations a progressive diminution of the arginase value occurred beginning between the second and fourth week of therapy. The reticulocyte, hemoglobin, and arginase response to vitamin B_{12} therapy of another patient with

typical pernicious anemia in relapse is shown in Fig. 2. This response typifies that of the other patients studied serially in this fashion. The first posttreatment arginase value was higher than the pretreatment level in 2 other patients who were followed serially.

Four cases with both subacute combined system disease and Addisonian pernicious anemia proved in each instance by histamine anacidity, megaloblastic bone marrow, and appropriate hematologic response to vitamin B_{12} were studied. Each had received continuous hematinic therapy for periods ranging from 2 to 6 years. All 4 patients yielded values (18.2, 11.2, 9.1, and 4.2) comparable to those of normal subjects.

Thalassemia: Five cases of thalassemia major showed a range of 55.7 to 114.9 with a mean of 84.6. All 5 of these determinations were obtained through the cooperation of Dr. Phillip Sturgeon. The essential data on these 2 groups of siblings is shown in Table II. It is of interest that fetal hemoglobin deter-

TABLE II. ERYTHROCYTE ARGINASE IN THALASSEMIA MAJOR

PATIENT	HEMOGLOBIN (GM. PER 100 ML.)	R.B.C. ARGINASE (MG. UREA N/10^{11} R.B.C.)
Es. C.	10.6	87.9
Ed. C.	9.4	85.3
T. C.	9.4	55.7
Ch. L.	8.8	114.9
Co. L.	9.2	79.2

minations were relatively higher in the first 3 patients than in the last 2, although all 5 were distinctly elevated. The parents of both groups of children had been previously studied and shown to possess a microcytic hypochromic blood picture consistent with thalassemia minor.

Twelve patients with thalassemia minor were included in this study. In all instances the red cell morphology by Wright-stained smears appeared hypochromic and microcytic. The majority of this group were of Mediterranean origin, and in several instances evidence of similar findings in other members of their immediate families was available. Red cell indices in all cases confirmed the hypochromic microcytosis, although the mean corpuscular hemoglobin concentration (M.C.H.C.) values were generally considerably higher relative to the mean corpuscular hemoglobin (M.C.H.) value. It was felt that in this respect the pattern of their indices was in distinct contrast to that of chronic iron deficiency anemia in which greater depression of the M.C.H.C. is usually seen. As shown in the scattergram, a considerable range exists between the maximum and minimum erythrocyte arginase values for patients with this condition. The lowest value was 13.5, and the highest was 89.2 with a mean of 36.3. Most values were therefore within the normal range, though the mean is somewhat high.

Iron deficiency anemia: Nine individuals with histories of recent or chronic blood loss or with anemias responding promptly to therapeutic iron were studied. The arginase values under these circumstances range from 2.7 to 22.0 with a mean of 11.5. The scattergram emphasizes that there is less spread

and a lower mean value when this group is compared with that of thalassemia minor. One patient was a 36-year-old woman with unexplained melena who presented with a hemoglobin of 5.9 Gm. per 100 ml. Her initial smear showed marked hypochromia and microcytosis, and the erythrocyte indices were calculated as M.C.V., 71; M.C.H., 18; and M.C.H.C., 26. The patient's bleeding ceased spontaneously, and in the absence of transfusions her hemoglobin returned to a value of 13.1 Gm. with a maximum reticulocytosis of 7.9 per cent on oral iron therapy over a period of 6 months. Her arginase value was 4.3. An 8-month-old male infant without evidence of blood loss but with an unequivocal dietary history of marked iron deficiency was studied when his hemoglobin value was 4.2 Gm. per 100 ml. Erythrocytes appeared markedly hypochromic and microcytic on smear. His erythrocyte arginase was 11.5. Five patients exhibited iron deficiency anemia as a result of gastrointestinal bleeding from duodenal ulcers, multiple telangiectases, and hemorrhoids. Their arginase values were 2.7, 8.8, 13.2, 18.3, and 22. One woman with long-standing hypoparathyroidism accompanied by an iron deficiency anemia showed an erythrocyte arginase activity of 15.5. In a child with a patent ductus arteriosus, whose serum iron was 16.5 μg, an arginase value of 6.8 was obtained.

Anemia of azotemia: In view of the findings of Liener and Schultze[15] that excessive liver arginase activity in uremic newborn rats could be reversed by the addition of vitamin B_{12} in vivo, examination of patients with uremia and/or chronic renal disease seemed pertinent. Five such patients were studied and showed arginase values ranging 5.3 to 11.3 with a mean of 8.5, as shown in the scattergram. The clinical diagnoses in this group included chronic pyelonephritis, chronic glomerulonephritis, arteriolar nephrosclerosis (2 patients), and malignant nephrosclerosis. All 5 patients were moderately anemic and all showed nitrogen retention. Their blood urea nitrogen values ranged between 31 and 168 mg. per 100 ml. The arginase activity of erythrocytes in patients with azotemia correlated with neither the severity of anemia nor degree of nitrogen retention.

Hemolytic anemia: Eight patients with various frank hemolytic anemias were studied. There were two cases of idiopathic acquired hemolytic anemia both of whom had previously been splenectomized, one of congenital hemolytic jaundice, two of paroxysmal nocturnal hemoglobinuria, one of nonspherocytic hemolytic anemia and porphobilinogenuria, one of a severe hemolytic anemia secondary to Hodgkin's disease, and one of a hemolytic anemia associated with myelofibrosis and myeloid metaplasia. Some of these patients had in the past required transfusions, but in no case had blood been given within a month of arginase determination. The possibility that a small proportion of the red cells sampled were foreign to the recipient must still be considered. The scattergram depicts the range of 0 to 30.9 found in the hemolytic anemias and the mean of 14.4 It is of note that one of the patients with idiopathic acquired hemolytic anemia, whose arginase value was 30.9, showed a reticulocytosis of 38 per cent. The patient with myelofibrosis and myeloid metaplasia, whose arginase was 11, was of unusual interest in so far as his

differential count revealed 396 nucleated erythrocytes per 200 white blood cells. It should not be inferred that the arginase activity of nucleated red cells is wholly included in the measurement of erythrocyte arginase, since the majority of the former cells are probably removed at the time of separation of the leukocyte plasma phase.

Leukemia: The erythrocyte arginase values of 9 cases of leukemia, all of which were anemic, are shown on the scattergram. One case of acute blastic leukemia with an arginase value of 12.9 is included. Three cases of chronic lymphatic leukemia were found to have arginase values of 11, 4.9, and 33.3. The 5 cases of chronic myelocytic leukemia were selected on the basis of peripheral smears, bone marrow picture, low leukocyte alkaline phosphatase value,[17] clinical course, and autopsy findings. Values for erythrocyte arginase of 57.1, 52.1, 44.3, 22.2, and 0 were found in this group of patients. Three of these values for chronic myelocytic leukemia are above the mean of normal subjects.

Polycythemia vera and myeloproliferative disorders: Polycythemia vera and certain myeloproliferative disorders not considered to be either polycythemia or leukemia are arbitrarily grouped together as a matter of convenience. Four cases were considered typical of polycythemia vera and showed arginase values of 59.1, 12.3, 8.7, and 7.1. The highest of these is noteworthy in that he exhibited the anemic and leukemoid phase of his disease at the time of the arginase determination, whereas 2 years previously he demonstrated the classical features of polycythemia vera and had, therefore, received internal irradiation with P^{32}. The other 6 patients include 3 of idiopathic myeloid metaplasia, 2 of myelofibrosis with myeloid metaplasia of the spleen, and one of osteosclerosis, resembling Albers-Schonberg disease. All 6 of these patients were moderately anemic. Their erythrocyte arginase values were 42.6, 29.9, 7.3, 24.9, 0, and 30, respectively. The erythrocyte arginase activities of this arbitrary group are shown in the scattergram.

Abnormal hemoglobins: Five patients with abnormal hemoglobins were studied. In addition one specimen of cord blood (ordinarily high in hemoglobin-F content) was obtained at the time of normal delivery. The hemoglobinopathies included 2 patients with sickle-cell trait (S-A), one with classical sickle-cell anemia (S-S), one with sickle-cell hemoglobin-C disease (S-C), and one with sickle-cell hemoglobin-D disease (S-D). The last mentioned patient has been reported separately by Sturgeon and co-workers.[17] His arginase value was 44.2, while those for the cord blood and the patients with sickle-cell trait, hemoglobin-S-C and homozygous-S, were 18.5, 1.8, 0, 34.5, and 16.6, respectively. These values can be seen on the scattergram.

Miscellaneous: The erythrocyte arginase values of 20 patients with various unrelated disorders are shown in the scattergram. The majority of these patients were mildly to moderately anemic. The highest value in the miscellaneous group (56.8) was obtained from a patient with Laennec's cirrhosis, jaundice, ascites, and anemia. Three cases of diabetes mellitus and 5 anemias of infection had normal arginase values. Other diseases in which normal

values for erythrocyte arginase were obtained include multiple myeloma, 2 leukemoid reactions, 4 cases of primary refractory anemia, acute intermittent porphyria, and metastatic hypernephroma. One patient in the miscellaneous group was selected for study because of a failure to absorb an oral tracer dose of CO^{60}-labeled vitamin B_{12}, probably conditioned by total gastrectomy 2 years earlier. Although this patient had not received vitamin B_{12} or folic acid prophylactically, no signs of anemia had developed. Moreover, his arginase value of 38.2 is comparable to that of normal subjects and falls below the minimum among patients with untreated megaloblastic anemia.

Leukocytes.—The results of leukocyte arginase determinations in various disorders are represented by another scattergram (Fig. 3). Normal subjects

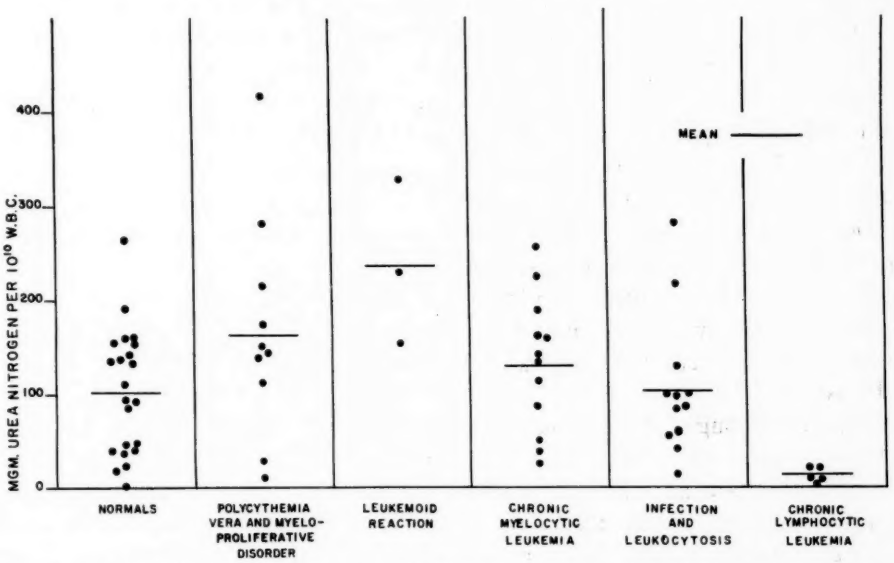

Fig. 3.—Scattergram of arginase activity of leukocytes obtained from normal human subjects and subjects with various disease states. Each dot represents a different patient. Values for arginase activity are expressed as milligrams of urea nitrogen liberated per 10^{10} leukocytes.

were selected in the same manner as for erythrocyte determinations. Leukocyte arginase values were expressed in terms of milligrams of urea nitrogen per 10^{10} W.B.C. The mean of 22 normal individuals was 103.4. The number of cases of leukemoid reactions were too few to justify the conclusion that the mean of 237 represented a significant increase over the normal. The scattergram emphasizes the wide spread of arginase values in normals, chronic myelocytic leukemia, polycythemia vera and other myeloproliferative disorders, infections, and other leukocytoses. No significant differences in leukocyte arginase activity among these disorders could be found. In addition one case of each of the following diseases failed to disclose a significant deviation from the normal: thalassemia minor, rheumatoid arthritis, infectious mononucleosis without lymphocytosis, Hodgkin's disease, and pernicious anemia. Two other cases of pernicious anemia revealed leukocyte arginase activities of 289 and

374. The very low values in all 5 cases of chronic lymphocytic leukemia are in distinct contrast to the other diseases plotted in the scattergram. In addition, 2 patients with acute blastic leukemia showed negligible leukocyte arginase activity. With the exception of these last 2 diseases, no consistent aberration from normal was observed in any of the disorders studied.

DISCUSSION

Whereas investigation of erythrocyte arginase activity showed significantly higher values in pernicious anemia, nutritional macrocytic anemias, and thalassemia major than in normal individuals and other types of anemia, no such difference was found for leukocyte arginase activity in various disorders. The most striking finding was the confirmation of the work of Kochakian and his co-workers,[7] in which a uniformly elevated erythrocyte arginase activity was found in pernicious anemia in relapse. We were able to add to this the impression that in other untreated nutritional macrocytic anemias some or all of the red cell population possesses increased arginase activity. In 2 patients with nutritional macrocytic anemia associated with regional enteritis this was found to be true. In these cases, however, there appeared to be a vitamin B_{12} deficiency, as is true in Addisonian pernicious anemia. Kochakian's work was further confirmed in so far as the initially high erythrocyte arginase activity returned to normal gradually as vitamin B_{12} administration induced hematologic improvement. In one patient the same sequence of gradual lowering of arginase activity during the first month was noted when folic acid was used instead of vitamin B_{12}. The effects of citrovorum factor were not investigated, although it is predicted that similar results would have also occurred with administration of this substance. The exact time of reversal of the elevated arginase activity toward normal after the initiation of vitamin B_{12} or folic acid is difficult to predict. This generally occurs between the second and fourth weeks. Some patients still showed slightly increased enzyme activity after one month of therapy. It is likely that these patients at the end of 6 months would have yielded normal values. Four cases of proved megaloblastic anemia, all treated satisfactorily for periods well in excess of one year, showed normal erythrocyte arginase activity. Disproportionate amounts of unit cell arginase in the total red cell population of an individual may explain the variability in the time required to restore the arginase value to normal. Erythrocytes formed during a deficiency of vitamin B_{12} appear to contain excessive amounts of arginase. Since the destruction and cessation of bone marrow release of such cells are not necessarily constant among different individuals with pernicious anemia receiving treatment with vitamin B_{12}, the amount of arginase activity after a constant time interval also varied with the patient. In 2 of the 7 patients that were followed serially, the erythrocyte arginase activity of the second specimen, drawn one week after onset of vitamin B_{12} therapy, exceeded the initial base-line value by 31 and 16 per cent. A similar phenomenon was not necessarily excluded in the other patients in so far as successive arginase determinations were relatively infrequent in most

of the patients studied. It can only be suggested that this increase in enzyme activity soon after the onset of therapy may have been related to a more rapid bone marrow release of abnormal cells which had undergone most of their medullary development prior to vitamin B_{12} therapy.

A positive correlation between the intensity of erythrocyte arginase activity and severity of anemia in terms of hemoglobin of venous blood is apparent only in cases of megaloblastic anemia. The highest value thus far encountered was noted in a patient whose hemoglobin was 2.7 Gm. per 100 ml. at the time of admission for megaloblastic anemia following resective and short-circuiting surgery for regional enteritis. Use of a suspension count of 500,000 R.B.C. per cubic millimeter rather than the customary one of about 1,000,000 provided an even higher final unit cell activity of 206 in this particular case. The erythrocyte arginase activity of the rest of the cases of pernicious and other macrocytic anemias tended to be higher when the degree of anemia was more severe. The most anemic of the 5 patients with thalassemia major in terms of hemoglobin provided the highest arginase value in this small group. Such a correlation could easily be due to chance. Certainly no consistent relationship between hemoglobin and arginase is evident in the other conditions studied.

Since it is not known whether the arginase present in red cells bears a relationship to stroma, hemoglobin, or some other moiety, final expression of enzyme activity in terms of a unit cell was elected. We recognize that the size of erythrocytes in pernicious anemia usually considerably exceeds that of the normal. This difference in size, however, could in no way account for the elevated arginase activity of patients with nutritional macrocytic anemias relative to that of normal individuals. In fact, the observations of Kochakian and his associates[7] are in close agreement with ours with respect to high erythrocyte arginase activity in pernicious anemia, despite several differences in method. Among these was the addition of a given volume of erythrocytes to the incubation mixture rather than of a given number of cells, as done in the present study.

The findings in Cooley's anemia were almost as striking as those in pernicious anemia. In both conditions a consistently marked elevation of arginase activity of red cells occurred in comparison to a group of normal patients and to several other anemias. The finding of elevated levels of this enzyme in the erythrocytes of 5 out of 5 cases of thalassemia major does not allow one to conclude that such an observation is true in every patient with this disease. Many additional determinations would be needed to settle this point. Unfortunately, the rarity of Cooley's anemia in the Los Angeles area is a hindrance to statistically significant figures. In contrast to thalassemia major, erythrocytes of patients with thalassemia minor showed a variable pattern of arginase activity, ranging from normal to a few distinctly elevated values. Because of the overlap erythrocyte arginase determinations as a clinical tool would not appear to offer any value in distinguishing between a severe thalassemia minor and very mild thalassemia major.

No explanation can be offered as to the reason for high arginase values in pernicious anemia and thalassemia major nor for the similarly high values in these two particular forms of severe anemia. A deficiency of vitamin B_{12} and related substances has never been demonstrated in thalassemia. There is a striking similarity in erythrocyte morphology and in resistance to iron therapy between thalassemia and the experimental anemia in pyridoxine-deficient animals.[18] Vitamin B_6, however, has been administered to patients with thalassemia major without hematologic benefit.[19] Experimental pyridoxine-deficiency anemia in humans has been produced only with great difficulty.[20] More recently a well-documented instance of severe anemia responding to parenteral vitamin B_6 has been reported.[21] Certainly a relationship between the arginine-ornithine-urea cycle and pyridoxine has been suggested in the work of McHenry and his collaborators.[22] They have repeatedly found that the fasting blood urea level is abnormally high in vitamin B_6-deficient rats. Of equal significance is the observation of Liener and Schultze,[15] who found that vitamin B_{12} seemed to bear a similar relation to urea formation. The offspring of rats reared on a diet of crude plant material were uremic at birth but could be cured by the addition of vitamin B_{12}. It has been suggested that both of these observations support a view that both vitamin B_{12} and vitamin B_6 enhance the utilization of circulating amino acids for protein synthesis. Of particular interest is the observation that Liener's uremic rats also demonstrated distinctly elevated hepatic arginase values before they received vitamin B_{12}.[15] However, while both vitamin B_{12} and pyridoxine deficiency appear in some experimental situations to result in increased blood urea values and while arginase is an important enzyme in the metabolism leading to urea formation, no reason for high arginase values in these two types of anemia is clearly present. *While certain similarities exist between thalassemia and pyridoxine deficiency anemia, it should be emphasized that no relation of pyridoxine to the defect in thalassemia has ever been demonstrated.* It is of interest, however, that of the wide variety of anemic states studied for arginase activity, only anemias due to vitamin B_{12} deficiency and the anemias of thalassemia show the marked departure from normal.

In view of the morphologic similarity between the erythrocytes of thalassemia and those of iron deficiency states, it was considered important to assay arginase activity in both of these groups. The normal or possibly low values of iron deficiency are in contrast to the elevated levels of thalassemia major and the sometimes elevated levels of thalassemia minor. The complexity of the method for determining arginase does not justify proposing its use as a tool in the differential diagnosis of thalassemia and iron deficiency.

Although it was not possible to study any patients exhibiting hemoglobinopathies involving combinations of other abnormal hemoglobins with the thalassemia gene, erythrocyte arginase determinations were done on cord blood, homozygous sickle-cell, sickle-cell hemoglobin-C, and sickle-cell hemoglobin-D bloods. The only hemoglobinopathy showing an elevated arginase value was sickle-cell hemoglobin-D disease, and this was only very moderate. The rarity

of this disorder would make further confirmation of this finding in other patients difficult. Because of the similarity of morphology between hemoglobin-H disease, thalassemia minor, and iron deficiency anemia, erythrocyte arginase assays in the former disorder would be of interest. The original description of hemoglobin-H disease characterizes this condition as a hypochromic microcytic anemia, refractory to iron therapy, with reticulocytosis, splenomegaly, decreased osmotic fragility to hypotonic saline and diminished red-cell survival.

Other conditions in which erythrocyte arginase was found to be slightly higher than normal were chronic myelocytic leukemia and the anemia of chronic liver disease. Three of the 5 patients with chronic myelocytic leukemia that were studied showed somewhat elevated values. None of these patients were under treatment with antifolic acid agents. An explanation for this observation cannot, therefore, be readily made. It is possible that a defect in either folic acid or vitamin B_{12} metabolism existed in the patient with anemia of liver disease. Only one other similar case was studied, and in this particular instance, the erythrocyte arginase value was normal.

The results of leukocyte arginase assays in various hematologic disorders were much less remarkable than those of erythrocyte arginase. It would appear from our data that both normal and diseased leukocytes possess definite arginase activity. In no particular disorder did leukocyte arginase appear markedly or consistently elevated above that of normal controls. In chronic lymphatic leukemia, infectious mononucleosis, acute leukemia, and the terminal blastic phase of chronic myelocytic leukemia, leukocyte arginase values were lower than normal. From these findings it can reasonably be inferred that myeloid, as opposed to mononuclear or blast cells, contribute primarily to the total arginase activity of leukocytes.

SUMMARY AND CONCLUSIONS

The arginase activity of erythrocytes and leukocytes in normal and diseased humans was assayed by a method employing L-(+)-arginase as the substrate and pure urease to convert urea to ammonium carbonate so that the latter might be measured spectrophotometrically after nesslerization.

Both erythrocytes and leukocytes possess arginase activity, the latter possessing 50 times as much activity per mean unit cell as the former. A considerable spread in the values for both cells was found in the normal subjects that were studied.

Erythrocyte arginase studies were of greater significance than leukocyte studies. Distinctly elevated erythrocyte arginase values were consistently found in patients exhibiting pernicious and other nutritional macrocytic anemias in relapse and thalassemia major. A similar consistent elevation was not observed in anemias of iron deficiency, azotemia, leukemia, inherited hemoglobinopathies, malignancies, infection, and of several other diseases. This method because of its complexity is not recommended in preference to other existing laboratory procedures for establishing a diagnosis of pernicious anemia or thalassemia major.

No distinctive pattern of leukocyte arginase values in various diseases affecting white cells was noted. When the major portion of the differential white count was replaced by nonneutrophilic leukocytes, arginase activity was lower than normal.

The role of arginase in blood corpuscles remains unknown. Its possible relationship to pyridoxine and cyanocobalamin is discussed.

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THE OXYGEN SATURATION OF BLOOD IN THE VENAE CAVAE, RIGHT-HEART CHAMBERS, AND PULMONARY VESSELS OF HEALTHY SUBJECTS

BRIAN G. BARRATT-BOYES, M.B., CH.B.,* AND EARL H. WOOD, M.D., PH.D.**
ROCHESTER, MINN.

FOR the detection and localization of arteriovenous shunts within the heart and associated great vessels, accurate definition of the degree of oxygen saturation of venous blood within the right chambers of the heart and great vessels in healthy subjects is essential. Previous investigators[1-4] have pointed out the marked variability in the oxygen content of blood withdrawn from the right atrium and to a less extent the right ventricle. This has been attributed to (1) variation in the subject's physiologic state with a resultant change in cardiac output in the interval between the collection of blood samples; and (2) laminar flow within the right-heart chambers resulting in incomplete mixing of streams of blood of different oxygen saturations from the superior and inferior venae cavae and the coronary sinus.

Because of this variability in oxygen saturation of venous blood within the normal heart, a method of withdrawing repeated blood samples in rapid succession through a cuvette oximeter, an instrument of high relative accuracy,[5,6] has been developed for use during cardiac catheterization in an attempt to reduce the effects of incomplete mixing and changing cardiac output to a minimum.[7,8] This presentation is an analysis of the data on oxygen saturation of blood obtained in this manner in an adequate series of unanesthetized, ambulatory, healthy, adult subjects. In addition to defining the mean and ranges of oxygen saturation within the right chambers of the heart and great vessels and the differences in the oxygen saturation of blood from these various sites, it has been possible to make some inference as to the relative importance of changes in cardiac output and laminar flow (incomplete mixing) on the variability of the values obtained at these various sites.

The data on oxygen saturation of blood herein reported were obtained during cardiac catheterization procedures, which also included recording of pressure, the inhalation of 95 per cent oxygen and, in some subjects, a period of exercise, the results of which are reported elsewhere.[9,10] The subject numbers used in this and related publications are identical.

METHOD

Twenty-six healthy subjects have been included in this study. Data are arranged according to age of the subjects in Table I. Fifteen of these subjects were healthy male

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*Fellow in Surgery, Mayo Foundation. The Mayo Foundation, Rochester, Minn., is a part of the Graduate School of the University of Minnesota.
**Section of Physiology, Mayo Clinic and Mayo Foundation.

physicians and 11 were patients in whom cardiac catheterization was performed to exclude cardiac abnormalities. Subjects 7, 24, and 26 were women, the remainder men. A detailed description of the technique of cardiac catheterization used has been reported elsewhere.[9, 11] The subjects had a light breakfast and received codeine, 0.5 gr. (0.032 Gm.), and seconal, 1.5 gr. (0.1 Gm.), 15 to 30 minutes before the procedure commenced. The cardiac catheter was connected to both a cuvette oximeter for whole blood and to a strain-gauge manometer by means of a 3-way stopcock.[12] This assembly was kept filled with heparinized saline solution. Samples of blood were obtained from the catheter by applying manual suction to a syringe connected to the cuvette oximeter. Two or more samples were obtained in rapid succession in this manner through the catheter-cuvette system; following this the system was flushed with heparinized saline solution to prevent clotting. The position of the tip of the catheter was rapidly changed under fluoroscopic control between each sample. Spot roentgenograms were taken in many cases for later confirmation of the position of the tip of the catheter. Pressure pulses were also continuously monitored.

Samples of blood obtained in rapid succession without the introduction of saline solution between individual samples were separated in time by a maximum of 1 minute and have been called "consecutive." Samples which were separated by the introduction of saline solution into the system have been called "random" being separated in time by a minimum of 2 minutes. An attempt was made, particularly in the 11 patients to repeat each consecutive series of samples at least once, and when doubt arose as to the interpretation of the saturations obtained, 2 or more times. In the majority of the subjects the blood withdrawn through the cuvette oximeter was discarded. In some, however, the blood was reinfused through the catheter-cuvette assembly immediately after withdrawal of each series of samples was completed.

The oxygen saturation of arterial blood was recorded both continuously by oximeters attached to one or both ears,[13] and intermittently from samples withdrawn through an additional cuvette oximeter attached to a needle indwelling in the right radial artery.

The galvanometer deflections produced by the cuvette and ear oximeters were observed visually by the use of the single scale circuit and both single and double scale deflections were continuously recorded photokymographically.[14, 15] Subsequently a calibration curve was constructed for each subject which related the single scale galvanometer deflection to the oxygen saturation as obtained by Van Slyke[5] analysis.

Samples of blood were withdrawn simultaneously from the radial and pulmonary arteries with the subject breathing air and also when breathing 95 per cent oxygen and analyzed for oxygen content within 30 minutes by the method of Van Slyke and Neill.[16] The oxygen capacity was determined after equilibration by the method of Sendroy[17] as modified by Roughton and associates.[18] In addition, in most subjects a sample was obtained for analysis from the site of lowest oxygen saturation to assist in the calibration of the cuvette oximeters.

Ninety-five per cent oxygen was administered by a closed circuit spirometer system during the latter part of the procedure on all subjects, except Subject 25, for an average period of 78 minutes. Dye-dilution curves were recorded photokymographically by the earpiece oximeters and by the cuvette oximeter attached to the needle in the radial artery[19] following the injection of Evans blue dye at multiple central sites[20] soon after the subject began breathing oxygen. Because of the apparent depression in the oxygen saturation of blood (as recorded oximetrically) which was produced by the circulating dye, the absolute values for oxygen saturation with the subject breathing oxygen were not suitable for analysis. The differences in oxygen saturation between consecutive samples of blood obtained under these conditions were not disturbed and, therefore, have been included.

RESULTS

The oxygen capacities and oxygen content of blood determined by analysis of samples simultaneously withdrawn from the radial and pulmonary artery

are given in Table I for each subject both while breathing air and while breathing oxygen. Other vital statistics including metabolic rate, cardiac output, and intracardiac pressures are reported elsewhere.[9] The average cardiac index for the group was 3.5 (2.8 to 6.3) L./min./M.^{2}

The oxygen capacity was normal in all subjects, averaging 19.7 vol. per cent with the subject breathing air with a range of 17.7 to 21.6 vol. per cent.[21] The average capacity of 19.6 vol. per cent while the subjects were breathing oxygen did not differ significantly from that while they were breathing air ($p < 0.1$). The mean total oxygen content of the systemic (radial) arterial blood (with the subject breathing air) was 19.4 (17.4 to 21.7) vol. per cent, and of the mixed venous (pulmonary artery) blood 15.3 (12.1 to 17.2) vol. per cent. While the subjects were breathing 95 per cent oxygen, these values were 21.2 (18.9 to 23.6) vol. per cent and 17.1 (13.4 to 19.4) vol. per cent, respectively. The arterial-venous difference in oxygen content of blood while the subjects were breathing air averaged 4.1 vol. per cent and did not change significantly during inhalation of 95 per cent oxygen.[9] The content of physically dissolved oxygen within the arterial blood (oxygen content minus oxygen capacity) during inhalation of 95 per cent oxygen averaged 1.6 vol. per cent and ranged from 1.0 to 2.6 vol. per cent.

The oxygen saturations, obtained by cuvette oximeter, of arterial (radial artery) blood and of venous blood withdrawn from the right-heart chambers and great vessels are also given in Table I together with the saturations of the samples from the pulmonary artery wedge. The cuvette oximeter values given in Table I are the averages of the several samples withdrawn from each site in individual subjects while breathing air, the means and standard deviations being calculated from these values. However, the ranges given in the text and table are based on all the individual samples obtained. The mean and range of the oxygen saturation of arterial blood equalled 97.3 (95 to 99) per cent.

Samples of blood from the inferior vena cava were withdrawn with the catheter tip at or just above the level of the diaphragm, the catheter having been first advanced to below the diaphragm to confirm its position in the inferior vena cava and then withdrawn slightly. Samples from this position, obtained in 19 subjects, showed a mean and range of oxygen saturation of 83.0 (76 to 88) per cent. The mean and range of the oxygen saturation of superior vena caval blood when samples were withdrawn with the catheter tip 1 to 4 cm. above the right border of the cardiac silhouette as seen fluoroscopically were 76.8 (66 to 84) per cent, a figure significantly less than the inferior vena caval saturation.

The catheter position at the time of withdrawal of right atrial blood varied considerably. In most cases it lay midway along the right cardiac border either near the tricuspid valve or adjacent to the lateral wall of the atrium. An analysis of the differences in saturation of blood obtained from various sites within the atrium was not attempted. Occasional blood samples of very low oxygen saturation withdrawn from the right atrium with the tip of the catheter

TABLE I. BLOOD OXYGEN VALUES

SUBJECT	AGE (YR.)	PER CENT OXYGEN SATURATION (CUVETTE); AIR						
		RADIAL ARTERY	I.V.C.*	S.V.C.*	R.A.*	R.V.*	P.A.*	WEDGE*
1	13	97	88‡	81	83	83	82	92
2	17	98	86	76	81	§	80	100
3	18	98	84	79	80	77	§	§
4	19	96	85	81	83	80	81	98
5	20	98	79	77	78	78	77	‖
6	21	97	84	78	81	79	79	97
7	22	98¶	65‡	74	74	79	78	‖
8	24	95	—	79	77	77	77	95
9	26	97	81	74	78	76	76	100
10**	27	97¶	78‡	69	79	69	75	99
11**	27	98	81	80	82	83	80	100
12**	27	96	87	81	78	78	77	§
13**	28	96	83	78	80	80	79	99
14**	28	98	86	77	81	77	78	100
15**	29	97¶	—	§	§	§	75	—
16**	29	99	88	75	83	80	82	98
17**	30	98	80	71	75	70	—	—
18**	30	98	76	72	76	§	79	‖
19**	31	98¶	79	77	75	75	75	—
20**	31	98	82	67	78	79	78	100
21**	32	98	82	79	80	77	76	98
22**	34	97	87	82	84	84	83	94
23**	40	98	—	77	79	80	77	100
24	41	97	—	77	81	81	77	100
25**	42	97	84	81	83	84	84	—
26	44	96	82	76	79	80	76	99
Mean		97.3	83.0	76.8	79.5	78.5	78.4	98.2
S. D.		±0.9	±3.2	±5.2	±2.8	±3.8	±2.6	±2.4
No. of samples		26	26	49	70	43	59	25
Range of individual samples		95-99	76-88	66-84	72-86	64-84	73-85	90-100

* = In this Table and in Tables II to V, I.V.C. indicates inferior vena cava; S.V.C., superior vena cava; R.A., right atrium; R.V., right ventricle; P.A., pulmonary artery; and wedge, wedge position in pulmonary artery.

†O_p = Physically dissolved oxygen (oxygen content − oxygen capacity) in arterial blood with subject breathing 95 per cent oxygen.

‡ = Samples obtained below diaphragm level, excluded from averages.

§ = Samples obtained while breathing 100 per cent oxygen only.

‖ = No flow from wedge position.

¶ = Earpiece oximeter readings.

** = Male physicians.

in a position which by fluoroscopy was apparently near the coronary sinus ostium were excluded. The mean and range of the oxygen saturation of right atrial blood excluding these samples equalled 79.5 (72 to 86) per cent, a figure intermediate between the inferior and superior caval blood saturations.

Right ventricular blood was withdrawn from both low in the right ventricle (adjacent to the tricuspid valve and from the main ventricular chamber) and high in the right ventricle (right ventricular outflow tract) and showed a mean and range of oxygen saturation of 78.5 (64 to 84) per cent, the mean value showing 1 per cent saturation less than that for right atrial blood.

The mean oxygen saturation of pulmonary arterial blood was 78 per cent. This value was similar to that of blood from the right ventricle; the range of 73 to 85 per cent was, however, smaller than the latter.

IN HEALTHY SUBJECTS

OXYGEN CAPACITY (VOL. PER CENT)		TOTAL OXYGEN CONTENT (VOL. PER CENT)				O_p† (VOL. PER CENT)
		RADIAL ARTERY		PULMONARY ARTERY		RADIAL ARTERY
AIR	95% O_2	AIR	95% O_2	AIR	95% O_2	95% O_2
17.7	17.8	17.4	19.2	13.6	15.8	1.4
20.3	20.4	20.3	22.1	16.1	17.6	1.7
19.1	19.3	19.0	21.9	—	17.9	2.6
20.0	20.2	19.3	21.2	15.9	17.8	1.0
21.5	21.7	21.7	23.6	17.2	19.4	1.9
19.9	20.1	19.8	21.9	16.0	18.3	1.8
—	18.7	—	20.0	—	16.9	1.3
21.6	21.1	20.9	23.2	17.0	19.3	2.1
18.2	18.2	17.9	19.4	14.0	15.3	1.2
—	20.2	—	22.0	—	17.5	1.8
19.0	18.8	18.9	20.6	15.0	16.2	1.8
19.0	19.0	18.4	20.4	15.3	17.3	1.4
19.3	19.3	18.8	20.8	14.7	17.2	1.5
20.9	20.7	20.9	21.9	16.5	17.5	1.2
—	22.4	—	24.3	—	—	1.9
20.7	20.7	20.8	21.9	17.1	18.4	1.2
18.2	18.4	18.0	19.6	12.1	13.4	1.2
21.2	20.4	20.6	22.0	15.7	17.3	1.8
18.8	18.5	18.4	20.2	14.6	16.0	1.7
21.3	21.3	21.1	22.6	16.8	18.5	1.3
20.7	20.0	20.3	21.9	15.8	17.5	1.9
19.4	19.4	19.0	20.8	15.8	17.3	1.4
18.3	18.0	18.7	20.1	14.2	15.9	1.8
18.3	18.2	18.3	20.0	14.2	16.1	1.8
20.5	—	—	—	—	—	—
18.4	17.4	18.3	18.9	14.2	15.2	1.5
19.7	19.6	19.4	21.2	15.3	17.1	1.6
±1.2	±2.4	±1.0	±1.4	±1.3	±1.4	—

The lowest oxygen saturation of any sample of venous blood withdrawn from the heart or great vessels (excluding the coronary sinus and right atrium near the ostium of the coronary sinus) was 64 per cent (obtained from the right ventricle), the highest 88 per cent (obtained from the inferior vena cava). The range in oxygen saturation of individual samples in individual subjects was wide, the differences in saturations of samples withdrawn from similar sites varying from 12 per cent saturation in the inferior vena cava and the pulmonary artery to 20 per cent saturation in the right ventricle. The standard deviation of the average values from subject to subject was greatest for superior vena caval blood samples (±5.2 per cent) and least for pulmonary artery blood (±2.6 per cent).

The catheter tip was satisfactorily wedged in a peripheral branch of a pulmonary artery on 45 occasions in 22 of the 26 subjects while breathing air or 95 per cent oxygen. The criteria used in the selection of these wedge positions have been enumerated elsewhere.[9] Although an attempt was made to withdraw blood in every instance, this was successful in only 30 (66 per cent) of the instances in which the catheter was in the wedge positions. Of the 30 samples of blood obtained, a total of four (13 per cent) were less saturated with oxygen than the arterial blood. With the subject breathing room air

(Table I) 25 samples were obtained in 17 subjects, with an average oxygen saturation of 98.2 per cent. The saturations of 3 of these samples (90, 92, and 94 per cent) were below the range of arterial oxygen saturation encountered in healthy subjects. The arterial oxygen saturations recorded simultaneously by earpiece oximeter in these subjects were 95, 96, and 97 per cent, respectively.

An analysis of differences in oxygen saturation of venous blood withdrawn from the venae cavae, the right chambers of the heart, and the pulmonary artery is given in Table II, using only the differences between single pairs of blood samples withdrawn in rapid succession from different sites on the right side of the heart. By confining the analysis to consecutive samples, the subject's physiologic state was assumed to be stable during the collection of each pair of samples. Variations in the oxygen saturation of venous blood resulting from a change in cardiac output were probably minimized in this way, although variations due to sampling from streams of blood of differing oxygen saturations would presumably not be affected.

On the basis of samples withdrawn in rapid succession from the two sites, inferior vena caval blood was more highly saturated with oxygen than superior

TABLE II. AVERAGE AND RANGE OF DIFFERENCES IN PER CENT OXYGEN SATURATION OF INDIVIDUAL PAIRS OF SAMPLES OF BLOOD WITHDRAWN IN RAPID SUCCESSION FROM DIFFERENT SITES ON THE RIGHT SIDE OF THE HEART (HEALTHY SUBJECTS BREATHING AIR OR OXYGEN)

	I.V.C. −S.V.C.	R.A. −I.V.C.	R.A. −S.V.C.	R.A. −LOW R.V.	R.A. −P.A.	LOW R.V. −P.A.	HIGH R.V. −P.A.
Number of subjects	20	19	26	24	17	18	13
Number of differences	30	31	82	44	20	23	25
Mean	+5.7	−2.2	+2.9	0	−0.3	0	0
Range	−2 to +16	−11 to +6	−3 to +12	−9 to +10	−9 to +5	−3 to +4	−3 to +2
S.D.	±4.9	±4.3	±3.0	±2.9	±3.5	±1.7	±1.4
P value	< 0.001	< 0.01	< 0.001	< 1.0	< 0.7	< 0.9	< 0.9

vena caval blood by a mean of 5.7 per cent (S.D. ±4.9), a statistically significant difference ($p < 0.001$). Right atrial blood was less saturated than inferior vena caval blood by a mean of 2.2 per cent (S.D. ±4.3) and more highly saturated than superior vena caval blood by a mean of 2.9 per cent (S.D. ±3.0). These differences were also statistically significant ($p < 0.01$ and 0.001, respectively). Comparison of the oxygen saturation of right atrial blood with blood from low in the right ventricle and the pulmonary artery did not show a systematic difference between these sites. Similarly there was no systematic difference between the oxygen saturations of samples from low and high in the right ventricle when compared with those from the pulmonary artery. The range of differences in oxygen saturation between the pulmonary artery and right ventricle was less than between the other sites studied.

When the averages of two or more differences in oxygen saturation from paired consecutive samples of blood are compared (Table III), variations in

oxygen saturation due to a changing cardiac output are minimized, and variations due to laminar flow particularly should be reduced. This reduction in variability produces a proportionate increase in the ability to detect abnormalities in the oxygen saturation of blood withdrawn from these sites. The standard deviation of the *average* differences obtained when comparing the oxygen saturation of right ventricular outflow blood with pulmonary artery blood (±1.2) was little changed from the *single* differences between these sites suggesting that mixing of returning systemic venous blood is relatively complete when it reaches the right ventricular outflow tract and pulmonary artery. Conversely the reduction in the standard deviation of the average as compared to single differences between the oxygen saturation of right atrial blood and that withdrawn from the inferior vena cava, the superior vena cava and from low in the right ventricle suggests that mixing of blood from superior and inferior venae cavae and coronary sinus was incomplete when traversing the atrium and lower part of the right ventricle.

TABLE III. AVERAGE DIFFERENCES IN PER CENT OXYGEN SATURATION BETWEEN TWO OR MORE PAIRS OF SAMPLES OF BLOOD WITHDRAWN IN RAPID SUCCESSION FROM DIFFERENT SITES ON THE RIGHT SIDE OF THE HEART (HEALTHY SUBJECTS BREATHING AIR OR OXYGEN*)

	I.V.C. −S.V.C.	R.A. −I.V.C.	R.A. −S.V.C.	R.A. −LOW R.V.	HIGH R.V. −P.A.
Number of subjects	8	9	22	14	9
Mean	+5.9	−2.2	+3.2	+0.1	+0.1
Range	+1 to +15	−7 to +2	0 to +8	−2 to +4	−2 to +2
S.D.	±4.4	±3.1	±2.3	±1.6	±1.2
P value	< 0.01	< 0.05	< 0.001	< 0.9	< 0.8

*Insufficient paired consecutive samples were available from the right atrium and pulmonary artery, and the low right ventricle and pulmonary artery for comparison of average differences between these sites.

TABLE IV. DIFFERENCES BETWEEN THE AVERAGE PER CENT OXYGEN SATURATIONS AT SITES FROM WHICH MULTIPLE (RANDOM) BLOOD SAMPLES WERE WITHDRAWN WITH THE SUBJECT BREATHING AIR*

	I.V.C. −S.V.C.	R.A. −I.V.C.	R.A. −S.V.C.	R.A. −R.V.†	R.A. −P.A.	R.V.† −P.A.
Number of subjects	6	8	16	12	17	11
Mean	+5.2	−3.4	+3.0	+0.6	+1.5	+1.5
Range	+1 to +10	−9 to +1	−2 to +11	−1 to +5	−1 to +4	0 to +4
S.D.	±3.2	±2.9	±3.1	±1.8	±1.3	±1.2
P value	< 0.02	< 0.02	< 0.01	< 0.3	< 0.001	< 0.01

*The number of samples from each site averaged three (range: 2 to 6).
†Low and high right ventricular samples both included.

In Table IV an analysis of the differences between the average oxygen saturations of samples of blood obtained in random fashion has been made. In this table the oxygen saturation at a particular site was obtained by averaging the several samples withdrawn from that site while the subject was breathing air, the number of samples from each site ranging from 2 to 6 and averaging 3. The figures were closely comparable to the average of the differences between paired consecutive samples given in Table III. Sufficient data were available in this instance to compare the average oxygen saturation of blood withdrawn

from the right atrium with the combined samples from low and high in the right ventricle and also with the samples from the pulmonary artery. A small but statistically significant difference in oxygen saturation was demonstrated between the right atrium and pulmonary artery ($p < 0.001$) and the right ventricle and pulmonary artery ($p < 0.01$); pulmonary artery blood was less saturated than both right atrial and right ventricular blood by a mean of 1.5 per cent. However, when the oxygen saturations of right atrial and right ventricular blood (R.A.-R.V.) were compared in a similar manner, the mean difference of +0.6 per cent was not statistically significant ($p < 0.3$).

Variation in oxygen saturation of mixed systemic venous blood, presumably resulting in the main from a change in cardiac output in the interval between the collection of samples, was estimated in subjects with multiple samples from the pulmonary artery, assuming that blood from this site was a true sample of mixed venous blood. Thus, in 19 subjects with an average of 3 samples of blood from the pulmonary artery separated by an average interval of 37 (5 to 147) minutes, the mean of the maximal variation in oxygen saturation was 3.3 per cent and the range was 0 to 10 per cent. If it is assumed that the rate of oxygen utilization and the arterial oxygen saturation remained constant during these periods, these variations in oxygen saturation of mixed venous blood represent average maximal spontaneous variations of more than 17 per cent in cardiac output values as determined by the direct Fick method in these resting healthy subjects.

TABLE V. THE MAXIMAL VARIATION OF THE DIFFERENCES IN PER CENT OXYGEN SATURATION BETWEEN PAIRED CONSECUTIVE BLOOD SAMPLES AND BETWEEN RANDOM BLOOD SAMPLES IN SUBJECTS WITH 2 OR MORE PAIRS OF CONSECUTIVE SAMPLES FROM THE SAME SITES WHILE BREATHING AIR*

	SAMPLES	R.A.-S.V.C.	R.A.-LOW R.V.	R.V.-P.A.
Number of subjects		13	6	7
Mean	Consecutive	2.5	2.5	2.3
	Random	7.7	7.5	7.0
Range	Consecutive	1 to 5	0 to 6	0 to 5
	Random	0 to 17	0 to 11	2 to 14
S.D.	Consecutive	±1.4	±2.5	±2.6
	Random	±5.7	±4.1	±4.5

*The time interval between random samples averaged 22 minutes (range: 2 to 115 minutes).

The value of the consecutive method of withdrawing samples from separate chambers for comparison of the oxygen saturation used almost exclusively in this study was investigated by comparing the differences in oxygen saturation between paired consecutive samples of blood from separate chambers with the differences between samples withdrawn from these sites in random fashion (Table V). As few samples were deliberately withdrawn in random fashion, samples from separate consecutive series were compared as random samples. Accordingly, in subjects with 2 or more pairs of consecutive samples from the same 2 chambers, the maximal variation for each of these differences was calculated; for example, with 3 pairs of consecutive samples from the right atrium and superior vena cava having oxygen saturations of 76 and 72, 80 and 78,

71 and 70 per cent, respectively, the differences between these paired consecutive samples equal +4, +2, +1; the differences between these samples compared in random fashion, however, equal –2, +6, +8, +10, –1, and –7; the maximal variation of the differences between consecutive samples would equal 3 per cent saturation, and between random samples 17 per cent. The interval between withdrawal of the consecutive blood samples included in Table V was 30 to 60 seconds and between the random samples 22 (2 to 115) minutes. One third of the random samples were separated by 2 to 8 minutes. The mean of the maximal variation of differences in samples obtained consecutively varied from 2.3 to 2.5 per cent at different sites, and was much less than that for samples obtained in random fashion of from 7.0 to 7.7 per cent saturation. The widest range of this maximal variation in consecutive samples was 0 to 6 per cent as compared to 0 to 17 per cent for random samples. No difference in variability was found between random samples separated by 8 minutes or less and random samples separated by more than 8 minutes.

COMMENT

The validity of the present analysis of oxygen saturation of blood as representative of healthy human beings rests on the absence of cardiovascular abnormalities, particularly intracardiac shunts, in the subjects included. Small intracardiac shunts cannot be satisfactorily excluded by the absence of any abnormality on clinical, electrocardiographic, or roentgenologic examination of the subject. Undetected veno-arterial (right-to-left) shunts, although decreasing the oxygen saturation of the arterial and mixed venous blood, would not appreciably distort the comparative oxygen saturation of blood withdrawn from various sites in the right chambers of the heart or great vessels. Should an undetected arteriovenous (left-to-right) shunt be present, however, the differences in blood oxygen saturation obtained at different sites by right-heart catheterization would presumably be appreciably distorted.

In this study indicator-dilution curves obtained after the injection of Evans blue dye (T-1824) at multiple sites within the right heart and great vessels were used to exclude hemodynamically significant vascular shunts. Curves of normal contour and time components were obtained in all subjects. Right-to-left shunts of a magnitude greater than approximately 3 to 5 per cent were considered excluded by this means.[22] Left-to-right shunts, recognizable by distortion of the disappearance slope of the dilution curves, are considered more difficult to detect than right-to-left shunts of similar magnitude.[23] However, in the presence of normal cardiac output and with an accurate system of continuous recording as in the present study, left-to-right shunts greater than approximately 10 to 15 per cent were also considered excluded.

Other cardiovascular abnormalities which might be of importance were considered excluded by measurements of intracardiac pressures and blood flow (reported elsewhere[9]) which fell within the normal range in all cases.

The accuracy of the cuvette oximeter in determining the oxygen saturation of blood has been previously reported. The absolute accuracy of the device varies with the oxygen saturation from ±1 to 4 per cent saturation.[6-8, 15]

The instrument has a high degree of relative accuracy, since under optimal conditions, the accuracy in the determination of differences in oxygen saturation has been reported to be ±0.1 per cent saturation.[5] Although the accuracy of the instrument decreases slightly when the lower oxygen saturations of venous blood are being recorded, this degree of accuracy is considered very satisfactory for purposes of routine catheterization.

The degree of accuracy obtainable in demonstrating arterialization within the right heart and associated great vessels by sampling of blood through a cardiac catheter is dependent on the constancy of the cardiac output during the collection of the samples and also on the degree of laminar flow (incomplete mixing) in the chambers from which the samples are withdrawn.

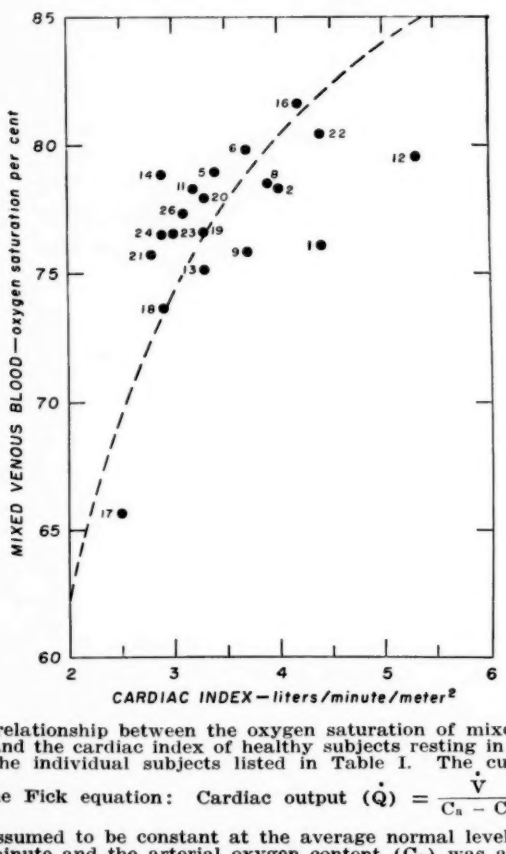

Fig. 1.—The relationship between the oxygen saturation of mixed venous blood from the pulmonary artery and the cardiac index of healthy subjects resting in the supine position. The numbers identify the individual subjects listed in Table I. The curved line was calculated on the basis of the Fick equation: Cardiac output $(\dot{Q}) = \frac{\dot{V}}{C_a - C_{\bar{v}}}$. The rate of oxygen uptake $(\dot{V})$ was assumed to be constant at the average normal level of 143 ml. per meter of body surface per minute and the arterial oxygen content (C_a) was assumed to be constant at the average normal level of 194 ml. per liter of blood (Table I).

An inverse relationship exists between the resting cardiac output and the oxygen saturation of mixed venous blood in the heart in the present subjects (Fig. 1). This relationship follows from the Fick equation $(\dot{Q} = \frac{\dot{V}}{C_a - C_{\bar{v}}})$, for when the rate of oxygen uptake $(\dot{V})$ and arterial oxygen content (C_a) are constant or near constant, as in the present subjects, the mixed venous oxygen content $(C_{\bar{v}})$ will vary reciprocally with cardiac output $(\dot{Q})$.[24-26] Under

the conditions of the present study the oxygen saturation of mixed venous (pulmonary artery) blood varied in oxygen content by as much as 10 per cent, presumably as the result of variations in the cardiac output and consequent changes in oxygen saturation of systemic venous blood, since laminar flow (incomplete mixing) is probably insignificant when returning venous blood has reached the pulmonary artery. These spontaneous variations in oxygen saturation of venous blood make it highly desirable that samples of blood withdrawn for comparison of oxygen saturations at different sites be separated by as short an interval as practicable in the effort to minimize these effects by decreasing the time available for the changes in cardiac output which evidently occur in subjects resting in the supine position.

On the basis of the data given in Table III, utilizing the mean and twice the standard deviation of the average differences between consecutive samples, the likelihood of exceeding a given range of variation in the oxygen saturation of blood from different cardiac chambers in healthy subjects can be expressed within 95 per cent confidence limits. Thus, there is a 95 per cent likelihood that the differences in caval saturations (I.V.C.-S.V.C.) will not exceed a range of −3 to +15 per cent saturation points, the right atrial and inferior vena caval saturation (R.A.-I.V.C.) a range of −8 to +4 per cent saturation points, and the right atrial and superior vena caval saturation (R.A.-S.V.C.) a range of −1 to +8 per cent saturation points. Therefore, with the consecutive method of sampling, a left-to-right shunt (arterialization) can be considered to be present in the right atrium when the oxygen saturation of right atrial blood consistently exceeds that of inferior vena caval blood by more than 4 per cent or that of superior vena caval blood by more than 8 per cent.

For the accurate detection of arterialization within the atrium a comparison of samples of blood from both cavae with those from the right atrium is desirable. The data indicate a 95 per cent likelihood of significant arterialization in the right atrium if the oxygen saturation of a blood sample withdrawn from the mid-right atrium exceeded the average saturation of samples withdrawn from the inferior and superior venae cavae by more than 4 per cent saturation. If 3 or more series of samples withdrawn in rapid succession from the inferior cava, mid-right atrium, and superior cava show that the saturation of right atrial blood exceeds the average saturation of inferior and superior caval blood by more than 2 per cent, there is a 95 per cent likelihood that there is a significant source of arterialized blood in the right atrium.

A comparison of the average saturation of inferior and superior vena caval blood with that of right atrial and right ventricular blood (Tables I and II) indicates that a simple arithmetic average of superior and inferior caval blood would give a closer approximation to mixed systemic venous blood than would the ⅓ to ⅔ ratio of weighting of superior and inferior vena caval blood[27] which has been recommended for calculation of left-to-right shunt in cases of atrial septal defect.[22]

Arterialization within the superior or inferior vena cava, the result of an anomalous pulmonary venous connection, should be suspected when the oxygen saturation of superior vena caval blood exceeds 84 per cent or that

of inferior vena caval blood exceeds 88 per cent, representing the upper limits of the normal ranges in oxygen saturation at these sites (Table I). By withdrawing consecutive blood samples every 1 to 2 cm. along the course of the vein, an abrupt fall to the normal caval saturation will localize the ostium of the vessel carrying pulmonary venous blood.

A 95 per cent likelihood of a left-to-right shunt at the level of the right ventricle is indicated (comparing consecutive samples, Table III) when the saturation of right ventricular blood exceeds that of the right atrium by more than 3 per cent on repeated sampling, and a shunt into the pulmonary artery is indicated when the oxygen saturation of blood at this site uniformly exceeds that of blood from the right ventricular outflow tract by more than 2 per cent. An increase of 2 to 3 per cent in saturation in the right atrium, ventricle, or pulmonary artery in the presence of normal saturation of pulmonary venous and systemic venous blood would be produced by a left-to-right shunt of 10 to 15 per cent of the total pulmonary flow. This is, therefore, the minimal left-to-right shunt which can be detected by the consecutive sampling method under these circumstances. A shunt of this magnitude in an average adult would correspond to a blood flow through the cardiac defect of more than 0.5 liter per minute. The data (Table V) indicate that the minimal left-to-right shunt detectable by the "random" sampling methods used in the usual diagnostic cardiac catheterization procedure would be greater than 20 per cent of pulmonary flow.

SUMMARY

Twenty-six healthy ambulatory subjects with cardiovascular respiratory systems normal in all clinical and laboratory aspects were investigated by cardiac catheterization. Multiple determinations of blood oxygen saturations were made in rapid succession by cuvette oximeter at various sites in the right side of the heart.

With the subject breathing air, the mean and range of the percentage oxygen saturation of radial arterial blood equalled 97.3 (95 to 99) per cent and of the venous blood withdrawn from the inferior vena cava, superior vena cava, right atrium, right ventricle, and pulmonary artery, 83.0 (76 to 88) per cent, 76.8 (66 to 84) per cent, 79.5 (72 to 86) per cent, 78.5 (64 to 84) per cent, and 78.4 (73 to 85) per cent, respectively, while that from the pulmonary artery wedge position equalled 98.2 (90 to 100) per cent.

The factors responsible for the variation in the oxygen saturation of venous blood were analyzed and methods suggested for reducing this variation.

The mean of the average differences in oxygen saturation between multiple paired samples of blood withdrawn in rapid succession from the right atrium and inferior vena cava, right atrium and superior vena cava, right atrium and lower part of the right ventricle, and upper part of the right ventricle and pulmonary artery equaled –2.2, +3.2, +0.1, and +0.1 per cent, respectively. With this method there is a 95 per cent likelihood that an abnormal source of arterialized blood (left-to-right shunt) is present within the right atrium when saturation of blood from it uniformly exceeds that in the inferior vena cava by

more than 4 per cent, that in the superior vena cava by more than 8 per cent, or an average of the inferior and superior caval samples by more than 3 per cent. In the absence of an atrial septal defect, arterialization in the right ventricle at the 95 per cent likelihood level can be considered to be demonstrated when its saturation exceeds that in the right atrium by more than 3 per cent and, in the absence of a ventricular septal defect, arterialization in the pulmonary artery is indicated when oxygen saturation of blood from it exceeds that from high in the right ventricle by more than 2 per cent.

Comparison of the average oxygen saturation of blood withdrawn from all positions within the right ventricle with those obtained from the right atrium and pulmonary artery was suggestive of a slight but significant reduction in the oxygen saturation of the venous blood during its passage through the ventricle. Streamline flow of blood from the coronary sinus and thebesian veins draining into the right ventricle was considered a likely explanation of this finding, although transient increases in cardiac output engendered by anxiety consequent to ectopic beats during manipulation of the catheter may be involved.

Variations in the oxygen saturation of multiple samples of mixed venous blood from the pulmonary artery from the same individual were consistent with the interpretation that the average maximal intra-individual variation in cardiac output, in these healthy subjects under conditions of supine rest, was ±17 per cent.

We wish to express our appreciation to Dr. H. J. C. Swan, Miss Lucille Cronin, and Mr. William Sutterer whose extraordinary efforts made this and the associated studies possible.[9, 10]

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DISTRIBUTION OF RADIOSULFUR, S^{35}

I. In the Human Adult

II. In the Young and the Adult Rat

Charles W. Denko, Ph.D., M.D.,* and Robert E. Priest, M.D.
Chicago, Ill.

STUDIES using radioactive sulfur, S^{35}, as a tracer have demonstrated that sulfated compounds exist throughout the body, being especially concentrated in the connective tissue. Previous investigators have noted the distribution of S^{35} in structures of the rat, human adult, hamster, mouse, and rabbit employing the techniques of quantitative S^{35} determination, radioautography, and tissue culture.[1-8] This uptake of S^{35} into the tissues is a vital process that is probably enzymatic in nature since it does not occur in boiled cartilage nor does it occur when pure chondroitin sulfuric acid is mixed with $Na_2S^{35}O_4$.[9] By isolating chondroitin sulfate containing S^{35} from the cartilage of animals receiving $S^{35}O_4^=$ intraperitoneally, Dziewiatkowski in the U. S. A., and Böstrom, in Sweden, have indicated that S^{35} is incorporated into chondroitin sulfate, the essential polysaccharide of the ground substance of connective tissue.[10, 11] As animals age this organic binding of radiosulfur by the tissues has been observed to decrease gradually but markedly.[7, 10]

This study was undertaken to delineate the distribution of S^{35} in various organs, especially the connective tissue, of a human adult. Studies were also done on the quantitative distribution of S^{35} in the organs and connective tissue of young and adult rats. Some interpretation of these data is presented.

EXPERIMENTAL

Part I.—The test animals were albino rats, young animals aged about 1 month and adult animals aged 5½ to 9 months. Data were obtained from 7 adult and 3 young animals. The rats received intraperitoneally 50 μc of S^{35} as $Na_2S^{35}O_4$ in saline in a single dose. Two adult animals were given this amount of sulfur in divided doses but did not show any appreciable difference in results when compared with animals given a single dose. The rats were killed by etherization 40 hours after receiving the sulfur. Tissues were removed, cleaned, and weighed. Those that were not processed on the day of sacrifice were kept in the Deepfreeze. In general, corresponding tissues were removed from each group of animals.

Details of the procedure for ashing tissues and precipitating the sulfur as barium sulfate have been outlined in a previous report.[13] Radioactivity determinations were carried out to a probable error of 3 to 5 per cent by means of a windowless gas-flow counter. Tissues that had fewer than 50 counts per minute per 100 mg. were counted to a probable error of 10 to 20 per cent.

From the Argonne Cancer Research Hospital and the Department of Medicine, The University of Chicago, Chicago 37, Ill.

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*Present address: Rackham Arthritis Research Unit, University of Michigan Hospital, Ann Arbor, Michigan.

Since all animals were not studied at one time, different lots of S^{35} were used.* The actual content was ±20 per cent of the stated activity. No correction was made for radiodecay. Because the body size of the young animals was markedly less than that of the adult animals the total radioactivity detected in the tissues is presented with a figure representing the comparison of the sulfur-35 found in each tissue to that amount present in costal cartilage, which is easily obtained as pure cartilage.

TABLE I. S^{35} FIXATION IN TISSUES OF AN ADULT MALE RAT AND A YOUNG MALE RAT

TISSUE	AGE 5½ MONTHS		AGE 1 MONTH	
	C.P.M.*	PROPORTIONAL†	C.P.M.	PROPORTIONAL
Cartilage				
Xiphoid	880	119	8,620	125
Costal	740	100	6,910	100
Articular (tibial cap)	390	53	5,330	77
Intervertebral disc	435	63	4,700	67
Tracheal (trachea)	365	49	2,700	39
Bone				
Membranous—skull	144	19	670	10
Cancellous—tibia	130	18	780	11
Aorta	230	31	1,960	28
Tendon	115	16	560	8
Skin	145	20	780	11
Kidney	167	23	530	8
Gastrointestinal tract				
Stomach—cardiac	175	24	830	12
Stomach—pylorus	125	17	360	5
Duodenum	96	13	340	5
Ilium	119	16	460	7
Colon	114	15	623	9
Eye				
Eye coats	157	21	700	10
Lens	3	< 1	70	1
Vitreous humor	0	0		
Lung	72	10	400	6
Spleen	61	8	310	4
Liver	30	4	110	2
Pituitary	110	15	850	12
Adrenal	70	9	220	3
Pancreas	24	3	275	4
Submaxillary gland	87	12	270	4
Testis	46	6	410	6
Prostate	44	6	83	1
Seminal vesicle	23	3	480	7
Blood clot	115	16	70	1
Cerebrum	21	3	130	2
Cerebellum	30	4	180	3
Spinal cord	50	7	470	7
Muscle, cardiac	50	7		
Muscle, skeletal	15	2		
Tooth	27	4	260	4
Fat	16	2	190	3
Lymph node			360	6

*c.p.m = counts per minute of S^{35} per 100 mg. of wet tissue.
†Proportional = relative distribution using costal cartilage fixation at 100.

Part II.—The patient observed was a 51-year-old white woman who entered the hospital with widespread carcinomatosis. Several years previously she had received arsenicals and penicillin as treatment for syphilis. Thirteen days before death she was given intravenously 2.0 mc. of S^{35} as $Na_2S^{35}O_4$ in saline. Tissue samples were removed at autopsy about 9 hours after death. These samples were kept in the Deepfreeze for several days before processing and counting. No correction was made for radiodecay.

*These were obtained from the AEC Oak Ridge National Laboratories, Oak Ridge, Tenn.

RESULTS AND DISCUSSION

Structures known to contain large amounts of sulfated mucopolysaccharides were found to have the highest concentration of S^{35}. In the rat (Table I) this group includes the cartilages of the xiphoid process, the ribs, the intervertebral discs, the trachea, and the articular cartilage of the tibia. Somewhat less S^{35} was found in the membranous and cancellous bones, in the aorta, and in tendon. In structures which contain considerable amounts of connective tissue, i.e., the kidneys and gastrointestinal tract, the concentration of S^{35} was of the same magnitude as the concentration in the bone. The eyecoats, skin, and pituitary gland, as far as observed concentration of S^{35} is concerned, can also be grouped with bone. The rest of the tissues, with few exceptions, had small amounts of the isotope.

Some differences appear in data obtained from the 5-month-old and 1-month-old rats. The decreased fixation of S^{35} by various tissues of adult rats was due in part to the difference in size—more radiosulfur being administered proportionately to the smaller animal—and in part to the decreased fixation of S^{35} due to aging. In some structures in the adult rat the amount of radiosulfur may reflect histologic changes in the tissues, e.g., the basement membrane in the kidney becomes thicker as the animal ages. Thus the kidney reflects a higher proportional activity in sulfur uptake. Similarly, the gastrointestinal tract of the adult animal has a greater amount of radiosulfate. It is interesting to note that the seminal vesicle of the young animal has more radioactive sulfur than the seminal vesicle of the older animal, while the reverse is true of the prostate gland. These data may indicate that the biochemical changes of aging have a greater effect in some tissues than in others. The aorta in the adult and in the young animal has approximately the same sulfur-^{35} content while skin and tendon in the older animal contain appreciably more S^{35} than skin and tendon in the younger rat.

These rat data cannot be compared rigidly to values previously reported since experimental conditions differed,[1, 2] especially in the time after injection of S^{35} that tissue samples were obtained. However, there is general agreement that the soft tissues are relatively low in S^{35} content. The high level of radiosulfur in the cartilage as noted here is similar to that found by Dziewiatkowski on quantitative and radioautographic study.[3]

The radiosulfur content of various tissues of the human adult (Table II) was found to contrast somewhat with the amount of S^{35} in corresponding tissues of the adult rat. Differences in the rate or period of growth of these two organisms may account for the variations. The costal and intervertebral disc cartilages of the rat have extremely high radiosulfate concentration while the costal cartilages in the human adult have relatively low S^{35} concentration. Although the tracheal cartilage in the human adult has a high level of radiosulfate, comparison with the S^{35} content of rat tracheal cartilage is difficult since the entire trachea of the rat was used for assay.

In the human adult the structures that were growing most rapidly, i.e., the tumor tissue (carcinoma of the breast) have the highest concentration of radiosulfur. This observation contrasts with that reported by Lewison and

co-workers,[2] who noted no marked deposition of S^{35} in breast cancer biopsy specimens. The high level of S^{35} found in the red bone marrow relates to the finding that the myeloid series of cells in human bone marrow form radiosulfate-bearing compounds in their cytoplasmic granules in vitro.[14] As in the rat, the gastrointestinal tract of the human adult has a high radiosulfate deposition. It is significant, in this regard, that the mucosa of the human colon in vitro has been demonstrated to form sulfated mucoproteins containing S^{35}.[15]

TABLE II. S^{35} FIXATION IN TISSUES OF AN ADULT FEMALE

SAMPLE	RADIOACTIVITY (C.P.M. PER 100 MG. WET WT.)
Tumor—Carcinoma of the breast	
metastatic area in liver	276
metastatic area in liver	242
Bone marrow, red, rib	250
Mucosa, gastrointestinal tract	
rectal	187
stomach, antrum	124
stomach, fundus	84
esophagus	117
Cartilage	
tracheal	173
costal	120
costal (fibrous)	69
costal (very fibrous)	circa 39
Kidney	
pyelonephritic area	162
normal tissue	120
Aorta	152
Peritoneal effusion	150
Adrenal	131
Thyroid, gland	115
adenoma	91
Mucosa, bladder	115
Bone, cancellous, vertebra	114
cortex of rib	60
Liver, fatty	102
Pancreas	98
Lung	79
Spleen	69
Lymph node, mediastinal	61
Disc, intervertebral	60
Skin, abdomen	50
Heart muscle	50
Blood clot	$<$ 50
Skeletal muscle	$<$ 50
Spinal cord	$<$ 50
Brain	$<$ 50
Fat	$<$ 50

The high S^{35} content of cartilage is best noted in the human adult in the clear glistening tracheal cartilage while the costal cartilage which is fibrous, opaque, and brittle contains lesser amounts of S^{35}. In vitro studies have previously demonstrated a high uptake of radiosulfur by human cartilage.[16] In the pyelonephritic portion of the kidney, inflammation and scarring from the action of the infecting bacteria resulted in the formation of more connective tissue than was present in the uninvolved portions.

The skin and lungs have an additional compound, heparin, present in the mast cells, that is responsible for a portion of the radiosulfate content. Mast cells, scattered throughout many organs may account for a portion of the slight radioactivities present in such structures as the spleen, lymph node, and liver, since these cells are known to incorporate S^{35}.[17] In the hard outer covering of the eyeball is a keratosulfate that may add to the potential radiosulfate content of this structure.[18] The eyecoat also contains the iris and ciliary body, both active in S^{35} uptake.[8]

Radioactive sulfate is very likely incorporated into tissues by at least two mechanisms. One of these may be an enzymatic transulfation process involving the movement of radioactive sulfate into sulfated compounds that are already formed. The other mechanism involves the utilization of the injected radiosulfur in the synthesis of new sulfated compounds. The amount of S^{35} found in the various organs and tissues reflects the metabolic balance of the incorporation and degradation of radiosulfate compounds present at the time of death.

SUMMARY

The distribution of radiosulfate in the tissues of an adult human female has been studied following the intravenous injection of S^{35}-labeled sodium sulfate. The quantitative distribution of radioactive sulfur in the tissues of young and adult rats was determined following the intraperitoneal injection of $Na_2S^{35}O_4$. In general the structures known to contain sulfated mucopolysaccharides, especially the connective tissue containing chondroitin sulfate, had the highest concentrations of S^{35}. In the human adult the structures with the greatest level of radiosulfur were the rapidly growing tumor (carcinoma of the breast), red bone marrow, mucosa of the gastrointestinal tract, cartilage, kidney, and aorta. Appreciable levels of sulfur-^{35} were noted in bone and in the endocrine glands. Low amounts were found in the muscle, nervous tissue, and fat. In the rat the structures with high S^{35} deposition were cartilage, bone, aorta, and tendon. Other organs and tissues with appreciable amounts of S^{35} were kidney, skin, gastrointestinal tract, eyecoats, lung, and pituitary gland. Changes attributed to ageing were reflected by a decreased total radiosulfate in the tissues, and in some structures with increasing amounts of connective tissues, higher levels of S^{35} proportionally.

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A MODIFICATION OF THE LATEX-FIXATION TEST FOR THE STUDY OF RHEUMATOID ARTHRITIS

M. S. Rheins, Ph.D., F. W. McCoy, M.D., R. G. Burrell, M.Sc., and E. V. Buehler, M.Sc.
Columbus, Ohio

INTRODUCTION

RECENTLY, Plotz and Singer[1] have described and evaluated a so-called "latex-fixation" test as an additional serologic technique in the diagnosis of rheumatoid arthritis. An excellent correlation was found by these authors to exist between the results of this test and those observed with the sensitized sheep erythrocyte technique. The above findings, coupled with the ready availability of the test reagents (polystyrene latex particles, borate-NaCl buffer, and human gamma globulin) as well as the relative simplicity of performing the test, suggested that a further evaluation of this technique was warranted.

The purpose of the present report is to confirm and perhaps extend the observations of Plotz and Singer and to suggest a "drop" modification of the latex-fixation test which minimizes both the time required for performing the test and the quantities of reagents needed without sacrificing sensitivity.

MATERIALS AND METHODS

Latex-Fixation Tests.—The essentials of the technique as described by Plotz and Singer[2] will be reviewed so that a comparison can be made between this and the proposed "drop" modification.

Latex-Fixation Test (Plotz and Singer).—Two milliliters of polystyrene latex particles (Dow Chemical Co., 0.81 micron in diameter) are suspended in 20.0 ml. of water. This suspension is filtered through Whatman No. 40 paper and further adjusted with the borate buffer (pH 8.2 and containing 0.85 per cent NaCl) so that approximately 0.1 ml. of this stock latex suspension, when mixed with 10.0 ml. of buffer, will match a light transmittance of 7 per cent as measured by a spectrophotometer at 650 mμ with a red filter.

One-tenth milliliter of the stock latex suspension is mixed with 9.5 ml. of buffer and 0.5 ml. of 0.5 per cent human gamma globulin (prepared by diluting lyophilized Fraction II with the borate-NaCl buffer). The resulting preparation comprises the test "antigen."

Serum samples, initially diluted 1:20 with the buffer, are further diluted by twofold increments through 1:5,120. To 1.0 ml. of each serum dilution is added 1.0 ml. of "antigen." Included in each test is an "antigen" control containing 1.0 ml. of "antigen" and 1.0 ml. of buffer.

The reactants are thoroughly mixed, by shaking the tubes, and incubated for 2 hours in a 56° C. water bath. After light centrifugation (2,300 r.p.m. for 3 minutes), the

From the Departments of Bacteriology and Medicine, Ohio State University.
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tubes are read for flocculation (0-4+) and the last serum dilution exhibiting evidence of aggregation is considered the titration end point. Flocculation by a serum dilution of 1:20 or greater is considered a positive test.

"Drop" Latex-Fixation Test (Rheins et al.).—The preparation of the "antigen" employed in these laboratories is similar to that just described. The notable differences are the use of polystyrene latex particles having a diameter of 1.17 micra (although of somewhat larger diameter than the particles utilized by Plotz and Singer, little difficulty has been encountered thus far because of the greater size) and the preparation of the gamma globulin. Human polio immune globulin, containing approximately 150.0 mg. of gamma globulin per milliliter, is diluted 1:30 with the borate-NaCl buffer. This dilution of gamma globulin has proved satisfactory and is analogous to the concentration of protein recommended by Plotz and Singer.

One drop of test serum is added to 1.0 ml. of buffer to effect a 1:20 dilution of the serum. From this serum stock, doubling dilutions, 1:20 through 1:5,120, are prepared by the usual serial transfer in small tubes (12 × 75 mm.), each but the first containing 2 drops of buffer. The transferring is easily accomplished with capillary pipettes. Into each tube (containing 2 drops of a serum dilution) are introduced 2 drops of the "antigen." Each titration, in addition to an "antigen" control, is accompanied by a negative serum control. This is prepared by admixture of 2 drops of a 1:20 dilution of a known negative serum and 2 drops of "antigen." After shaking, the tests are incubated for 30 minutes at either 56° C. or 37° C. (water bath).

Experience has shown that extremely mild centrifugation affords the most readable tests. Routinely, in these laboratories, this has resulted from centrifugation at 1,000 r.p.m. in an angle centrifuge. As soon as this speed is attained, centrifugation is halted and tubes are read in a near horizontal position by the oblique light from either a shaded bulb or fluorescent desk lamp. Regardless of the conditions employed for examination, the titration end point (the last serum dilution in which aggregates are evident) is determined by comparison with the negative serum control. Differences in viscosity between the greater serum concentrations of the titration and the "antigen" control sometimes introduces an element of error into the test. This is overcome to a great extent by the rigid use of the negative serum control.

By the modification just described, it is possible to perform a complete titration with 1.0 ml. of "antigen" and one drop of test serum.

A screening procedure has proved practical when large numbers of sera are to be tested. Two drops of each serum stock (1:20) and 2 drops of antigen are mixed and tested as above. The appropriate controls are included and, after incubation and centrifugation, each serum is examined and the reactivity graded from negative (–) to 4+. All positive sera and those of doubtful reactivity are titrated. Since the first serum dilution in the titration is also 1:20, an opportunity is afforded to review the doubtful sera. Although partial zones are sometimes observed (the second or third serum dilutions showing greater reactivity than the initial 1:20 dilution), no evidence of full zones has been recorded. This phenomenon is being investigated and the results will be reported at a later date.

Globulin-Coated Tanned Erythrocytes.—One-tenth milliliter of washed, packed sheep erythrocytes are tanned by the method described by Rheins and Thurston.[3] To this quantity of tanned red cells is added 1.0 ml. of human polio-immune globulin, diluted with phosphate-buffered saline (pH 7.4) so that each milliliter contains 5.0 mg. per milliliter. After incubation at 37° C. (water bath) for 30 minutes, the globulin-coated erythrocytes are thrice-washed with the saline and prepared as a 0.5 per cent suspension.

All test sera are inactivated at 56° C. for 30 minutes and adsorbed twice at room temperature with 0.4 volumes of washed, packed normal sheep erythrocytes to remove naturally occurring antibodies (each adsorption 30 minutes).

Performance of Agglutination Test.—The test is performed with quantities of reagents identical with those described for the "drop" modification of the latex-fixation test, i.e., to 2 drops of each serum dilution (1:20 through 1:5,120) are added 2 drops of

the globulin-treated tanned erythrocytes suspension. After mixture and incubation at 37° C. for 30 minutes, the tubes are centrifuged (1,000 r.p.m. for 2 minutes) and examined for agglutination. The usual controls are included with each test and the titration end point is determined as the last serum dilution producing visible agglutination.

EXPERIMENTAL RESULTS

Validation of the "Drop" Modification of the Latex-Fixation Test.—In order to determine the reliability of a proposed "drop" modification of the latex-fixation test, the following experiments were conducted. (1) Sera, shown to be positive or negative by the macromethod, were titrated by the "drop" modification. In each instance, the incubation was at 56° C. for 2 hours. (2) Titrations of positive sera were conducted at 30 minutes and at 2 hours by the "drop" modification. (3) By this technique, positive sera were titrated at both 37° C. and 56° C. for 30 minutes. Many of the same sera were employed in the three experiments.

The composite results of these tests are recorded in Table I. In no instance was a variation in titer of more than 2 dilution tubes observed. Of particular interest is the absence of false reactions among the various procedures.

TABLE I. THE EFFECT OF VARYING THE QUANTITY OF REACTANTS, THE INCUBATION TIMES, AND TEMPERATURE ON THE ACCURACY OF THE LATEX-FIXATION TEST

SERA			METHOD		INCUBATION				
TOTAL NO. TESTED	POSITIVE	NEGATIVE	MACRO	"DROP" MODIFICATION	30 MINUTES	2 HOURS	INCUB. TEMP. 37° C.	INCUB. TEMP. 56° C.	PERCENTAGE AGREEMENT
40	20	20	40	40		×		×	100
25	25			25	×	×		×	100
22	22			22	×		×		100

Based on these results, the "drop" modifications as outlined in the previous section were employed in all subsequent experiments. Incubation of the tests was conducted at either 37° C. or 56° C., whichever was most convenient.

TABLE II. RESULTS OF TESTING VARIETY OF SERA BY LATEX-FIXATION TEST AND SENSITIZED, TANNED ERYTHROCYTE TECHNIQUE

TOTAL NO. OF SERA TESTED	NO. OF REACTIVE SERA	POS. BY LATEX-FIX. TEST	POS. BY SENS. TAN. R.B.C.	POS. BY BOTH TESTS	POS. BY LATEX-FIX. NEG. BY SENS. R.B.C.	POS. BY SENS. R.B.C. NEG. BY LATEX-FIX.	PER CENT AGREEMENT
125	58	47	51	40	7	11	69

Correlation Between Results of the Latex-Fixation Test and the Coated-Tanned Erythrocyte Technique.—One hundred twenty-five sera were employed

in this study. Since the purpose of the experiment was to compare the results obtained with the latex-fixation test and those observed with the globulin-treated tanned erythrocytes, sera were chosen at random from those available to these laboratories. Of the total sera tested (see Table II), 47 were positive by the latex-fixation test and 51 by the sensitized, tanned erythrocyte technique. Forty sera were positive by both tests. In 7 instances, sera were positive by the latex-fixation test and negative by the sensitized, tanned red cell technique. Eleven sera reacted only with the sensitized erythrocytes. Of the 58 sera which reacted positively in one or both tests, 69 per cent (40 sera) were in agreement.

In general, the titers observed with the latex-fixation test were higher than were those seen with the sensitized red cell technique. However, the converse occurred with sufficient frequency to merit investigation as to whether these discrepancies were attributable to the techniques or reflect differences in the mechanisms of the two tests.

Serologic Reactivity of Patients Ill With Rheumatic Diseases, as Well as Other Selected Conditions.—The sera from 105 patients with a clinical diagnosis of active rheumatoid arthritis were examined by the modification of the latex-fixation test described above (Fig. 1). Ninety-two (88 per cent) gave

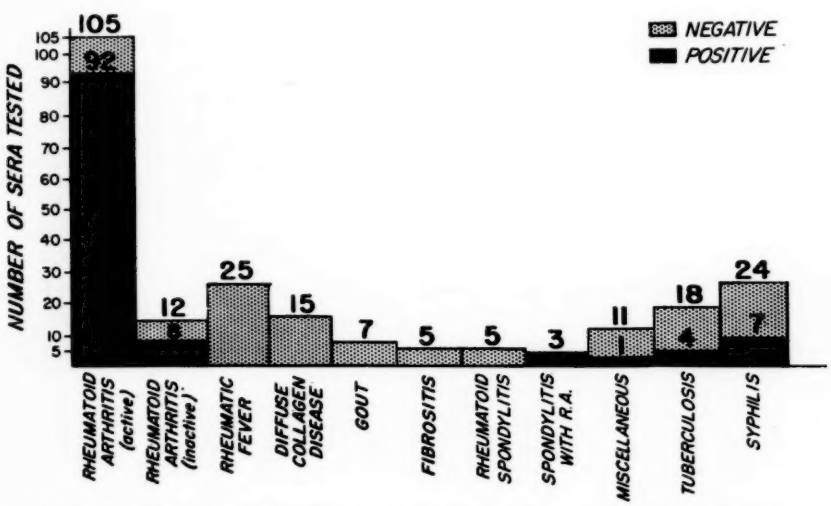

Fig. 1.—Latex-fixation reactivity of sera from patients ill with rheumatic and other selected diseases.

positive reactions. In patients who had either inactive rheumatoid arthritis clinically or were thought to be in remission because of therapy, the test was positive in only 8 (66 per cent) of the sera tested. The sera from those patients with rheumatoid spondylitis in conjunction with peripheral rheumatoid arthritis were uniformly positive (100 per cent). In patients ill with other forms of rheumatic fever, diffuse collagen disease (lupus erythematosus, scleroderma) as well as spondylitis, gout, and the fibrositis syndrome, the test sera were consistently negative.

It should be mentioned that the latex-fixation titers observed with the positive-reacting sera ranged from 1:20 through the greatest serum dilution, 1:5,120. Generally, the higher titers were observed in those sera from patients exhibiting more active disease.

In analyzing the rheumatic disease patients as a group, the test was positive in 103 of 120 patients (86 per cent) who were considered to have rheumatoid arthritis. In a variety of other clinical conditions, nonrheumatic in nature, there was one positive serum from a case of infectious mononucleosis, 4 positive sera from 18 cases of tuberculosis, and 7 positive sera from 24 cases of syphilis. It is interesting to note that the sensitized sheep cell agglutination test also is reported to give positive reaction in these illnesses (infectious mononucleosis, tuberculosis, and syphilis) in a certain percentage of cases.

DISCUSSION

In attempting to correlate the serologic results with the classification of patients with rheumatoid arthritis the following diagnostic problems were encountered. The majority of sera from patients who presented the minimal criteria required for diagnosis of probable or possible rheumatoid arthritis (as outlined by the Committee on Diagnostic Criteria of the American Rheumatism Association) gave negative reactions. Furthermore, some of these patients were under active treatment and it was difficult to evaluate the degree of activity of their disease at this time. The testing of such sera as these may have resulted in false negative reactions, thus lowering the over-all percentage positivity.

Approximately 50 per cent of the sera from patients who satisfied the criteria for definite rheumatoid arthritis, but at the present time presented no clinical evidence of active disease, reacted positively. This fact suggests that either the serologic demonstration lags behind the clinical course of the disease or that in a percentage of such individuals remissions are temporary. Conversely, it is extremely difficult to determine why some of the very active rheumatoid arthritis cases gave a negative reaction.

The most difficult problem in classifying some of the patients arose in the so-called diffuse collagen disease group. These represented patients who satisfied five of the criteria for definite rheumatoid arthritis, but in addition exhibited other systemic involvement which made the diagnosis of diffuse connective tissue disease the only acceptable clinical impression. The single common denominator in this group was the over-all negative reactivity of their sera.

The significance of the positive reaction in the other unrelated clinical disorders is not known.

From the analysis of the data presented in this preliminary report, the following conclusions seem warranted.

The "drop" modification of the latex-fixation test agrees with the results of the sheep cell agglutination test with regard to specificity. Also, the ease

of performing the test is an added advantage and, in addition, this test requires less technical time and fewer reagents.

In view of these observations, it is suggested that this test merits further evaluation as a serologic tool in the diagnosis, long-term management, and prognosis of rheumatoid arthritis.

The problem of reactive sera from those individuals ill with diseases other than rheumatoid arthritis suggests further investigation as to the identity of the factor or factors responsible. Furthermore, the relationship of these substances and those responsible for the positive reactivity of sera from rheumatoid arthritis patients should be established.

SUMMARY

A "drop" modification of the latex-fixation test for the serologic study of sera from patients ill with rheumatoid arthritis was described.

The results obtained with this modification apparently are in general agreement with those reported by other investigators who employed a macromethod.

It is suggested that this test merits further evaluation in the study of rheumatoid states.

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CHRONIC TOXICITY STUDIES OF HYDRALAZINE (APRESOLINE) IN DOGS WITH PARTICULAR REFERENCE TO THE PRODUCTION OF THE "HYDRALAZINE SYNDROME"

EDMUND L. DUBOIS, M.D., YALE J. KATZ, M.D., PH.D., VIVIAN FREEMAN, AND FRANK GARBAK, M.D.
LOS ANGELES, CALIF.

HYDRALAZINE (Apresoline) has been used in the treatment of hypertension since 1951. In 1954, two groups of workers independently described a febrile syndrome, occurring in patients taking this drug for many months, which was characterized by fever, malaise, arthralgia, abnormal liver function tests, lupus erythematosus-like rashes, and, in the occasional case, even L.E. cells.[1, 2] These symptoms subsided on withdrawal of hydralazine or upon the administration of adrenal corticoids while maintaining the patient on hydralazine. Others have also described isolated cases of this clinical picture. Comens[3, 4] administered this drug to 8 dogs and, in 1955, reported the development of a systemic lupuslike syndrome in animals receiving 10 to 12 milligrams per kilogram for periods of 1 to 8 months. Studies by Earl[5] at Ciba Pharmaceutical Products, Inc., were not confirmatory. Since the laboratory induction of systemic lupus erythematosus (SLE) might provide a useful tool in the study of this disease, we undertook the following experiments.

MATERIALS AND METHODS

Mongrel dogs weighing from 7 to 19 kilograms were fed hydralazine in doses of from 5 to 50 mg. per kilogram per day, 7 days a week. The dosages were determined by the control period weights throughout the study and were not revised downward when weight loss occurred with the exception of Dogs 2, 4, and 10 (Tables I and II) during the latter part of the experiment. Initially the drug was given the first few animals as sugar coated tablets for a short period, but more extensively the medication was administered in powder form. First the powder was mixed with approximately 2 ounces of horsemeat but more recently it was given in capsules or forcefed if required. When the animals became too violent to be fed the material, it was injected intramuscularly. The basic ration was Purina checkers.

Five types of L.E. cell preparations were performed at intervals of every few weeks, these consisted of the Comens technique of utilizing serum from the dogs plus leukocytes from heparinized human blood,[3] the sieved 2-hour clot method of Zimmer and Hargraves,[6] the Snapper-Nathan ring technique,[7] and the plain heparinized method.[8] Since the description of the rotary method by Zinkham and Conley[9] and the confirmation in this laboratory of its increased sensitivity over the plain heparin method we have employed it instead of the

From the Department of Internal Medicine, University of Southern California School of Medicine.

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TABLE I. SUMMARY OF THE COURSE

DOG	DURATION OF TREATMENT (MO.)	WT. LOSS* (KG.)	DOSE (MG./KG.)	DOSE (MG.)	TEMPERATURE†
1. Female chow (17.3 Kg.)	4.5	1.4	10-20	175-350	100.8-104.5
2. Female chow (17.0 Kg.)	12.5	3.4	10-20	170-350	102.2-102.2
3. Male wire-haired terrier (9.5 Kg.)	9.0	2.2	10-25	95-230	100.4-103.1
4. Male mongrel (10.9 Kg.)	9.0	3.2	15-20	150-200	100.7-102.2
5. Male Bedlington terrier (7.7 Kg.)	2.0	3.1	20	154	98.6-103.7
6. Female mongrel (18.6 Kg.)	1.2		25	465	101.5
7. Female mongrel¶ (13.2 Kg.)	0.5		35	465	
8. Female mongrel¶ (11.3 Kg.)	0.25		50	575	102
9. Female mongrel (11.8 Kg.)	2.0	1.4	10-15	118-160	101.8-101.5
10. Female mongrel (23.6 Kg.)	5.5	4.1	10-20	236-430	101.4-101.3

*Control and lowest level.
†Control level and maximum temperature.
‡Initial sedimentation rate (Wintrobe) and maximum.

unrotated heparin technique.[10] In addition, determinations of hemoglobin, hematocrit, sedimentation rate (Wintrobe), white blood cell, and differential counts were performed routinely. The amounts of blood drawn were limited to a maximum of 20 ml. every 3 weeks to minimize blood loss. In addition, routine urinalysis and in some cases blood urea nitrogen, bilirubin, phenolsulfonphthalein and renal biopsies were performed before and after treatment.

COURSE OF HYDRALAZINE TREATED DOGS

The pattern of toxic reactions varied directly with the dosages given. Five dogs, as outlined in Tables I and II, and Fig. 1, were initially started on 10 mg. per kilogram. (Table II shows the detailed studies performed on all animals which, for the sake of abbreviation, have been summarized in Table I). Four dogs tolerated this amount without difficulty and within 4 to 8 weeks the dosage was increased to 15 mg. per kilogram. The fifth animal, Dog 3 (Table I), became cachectic and anemic on only 10 mg. per kilogram within 5 weeks (Fig. 1). His medication was omitted for 10 days and then reinstituted at a level of 5 mg. per kilogram with gradual improvement in his physical condition and laboratory findings. He was finally able to tolerate a maximum of 25 mg. per kilogram for 5 weeks by gradually increasing the dose. Dog 9 (Table I) refused the medication 5 days after the dose was increased to 15 mg. per kilogram. She was forcefed and died 2 weeks later following 24 hours of status epilepticus. The other 3 animals, Dogs 1, 2, and

OF HYDRALAZINE TREATED DOGS

HGB.* (GM.)	SED.‡ RATE	HT.*	WBC§ (THOUS.)	REMARKS‖
12.5-10.5	6-44	44 -38	13.5-27.0	Preterminal convulsive seizures. Dog sacrificed 5 days later
12.0- 9.0	4-21	44 -30	11.7-24.4	See Table II. Several convulsive seizures. Well at present
13.0- 8.0	35-61	41.5-28	14.5-38.0	Preterminal convulsive seizures. Dog sacrificed. (See Fig. 1)
14.5- 9.0	2-53	50 -30	10.1-22.2	Convulsive seizures after 4 mo. of therapy and intermittently since. Cachectic at present
14.0- 3.8	12-83	44 -10	15.5-23.0	Progressive cachexia. Ten normoblasts per 100 WBC on smear. Died.
15.0-10.5	2-14	52 -34	19.0-19.2	Violent 1 week after start of medication. Sacrificed when moribund
13.0	9	45	19.9	Violent 10 days after start of medication. Died
12.0	8	44	18.0	Violent 4 days after start of medication. Died
12.0-11.0	6-26	44 -42	13.2-14.2	Preterminal convulsions. Died. Last hemogram done 3 weeks before death
14.0-13.0	6-2	49 -50	9.1-13.5	Convulsive seizures 3 mo. after start of medication. Doing well despite continued hydralazine

§Minimum and maximum levels.

‖Numerous L.E. cell studies by the five techniques described in the text were performed at monthly intervals. All were negative.

¶Control values only. Dogs were too violent to have repeat studies performed.

10, tolerated 15 mg. per kilogram for 5 to 7 weeks with only slight weight loss and anemia appearing in Dog 2 (see Table II). The dose was then increased to 20 mg. per kilogram. Within 4 days Dog 1 began to snap at the attendants and convulsed. He was sacrificed. Dog 2 after receiving 20 mg. per kilogram for 4 weeks refused his medication and had to be forcefed. Two weeks later he had convulsive seizures. Dog 10 had convulsive seizures 4 weeks after increasing the dose to 20 mg. per kilogram. The dose was temporarily reduced in Dog 2 as shown in Table II and then cautiously increased again to 20 mg. per kilogram. There have been no further seizures. Six weeks after increasing the medication to 20 mg. per kilogram a normocytic anemia of 9 Gm. per cent appeared but this corrected itself spontaneously 4 weeks later. Dog 10 had no further seizures despite continuations of the medication at the same level. She has not developed anemia.

Dog 4 (Table I) was started on 15 mg. per kilogram which he tolerated well for two months. Six weeks after increasing the medication to 20 mg. per kilogram he had a convulsive seizure and normocytic anemia. The drug was omitted for 2 weeks and then reinstituted at a level of 15 mg. per kilogram. During the past 3½ months he has again received 20 mg. per kilogram with partial correction of his anemia and no recent seizures.

TABLE II. COURSE OF DOG 2 (FEMALE CHOW)

DATE	WT. KG.	DOSE MG./ KG.	DOSE MG.	TEMP.	HGB. (GM.)	SED. RATE	HT.	WBC (THOUS.)	L.E. CELL PREP. CLOT	L.E. CELL PREP. WBC AND SERUM	L.E. CELL PREP. HEP.	L.E. CELL PREP. RING	REMARKS
2/ 6/56	17.0	10	170	102.2	12.0	4	44	12.8	0	0	0	0	Pyuria present
3/29/56	18.2	10	170	101.5	12.5	4	45	16.5	0	0	0	0	Urinalysis normal
4/ 7/56	16.8	15	260										
4/19/56		15	260										Medication given in meat
5/11/56	15.9	20	340	100.5	10.5		36	13.2					Bacilluria present
5/25/56	13.2	20	350	100.7	10.0			24.4					Dog quiet
6/ 7/56		20	350										Refused horsemeat & medication. Forcefed for 2 days.
6/16/56	13.6	20	350	100.5	10.0	21	31	20.1	0	0	0	0	Convulsive seizure. Bacilluria present
7/16/56	15.0	20	350	101.5	9.0		30	20.0					Doing well
8/ 2/56	17.7	20	350	101.5	12.0					0		0	Rotary L.E. = 0
8/13/56		20	350										Discontinued meat*
8/28/56	11.7	0	0		10.0	15	30	14.5		0			Rotary L.E. = 0. Convulsion
9/ 5/56		20	350										
9/ 9/56	10.4	0	0		9.0							0	Convulsion
9/24/56		15	260		11.0	15	36	14.0				0	Rotary L.E. = 0 Restart hydralazine
10/ 5/56	15.4	15	260	102.2									Doing well
10/16/56	15.0	13	225	102.2	11.0	8	38	15.3					Doing well
10/26/56	14.5	13	225	102.2									Eating poorly
11/ 2/56	15.0	15	225	100.5									Eating well. Urine urobilinogen pos. 1-20; neg. 1-40. Bile neg.
11/12/56	14.5	15	225	100.5									
11/26/56	13.6	15	210	100.5									
12/ 3/56	13.6	20	270	100.4	12.5		40	11.7	0	0		0	Urine normal. Bile = 0 Doing well
12/17/56	13.6	20	270	100.4									Doing well
1/17/57	13.6	20	270	100.4	9.0	7	31	16.1		0		0	Rotary L.E. = 0 Doing well
2/15/57	13.6	20	270	100.4	12.0		40	11.2					Rotary L.E. = 0 Alive and well

*Comens suggested, in a personal communication, that since hydralazine was a chelating agent, the medication be given without meat.

It seems that tolerance to the drug was acquired by cautiously increasing the doses as shown in these 6 animals. The use of larger initial amounts discussed below produced a rapidly toxic downhill course without giving the animals time to become accustomed to the drugs.

Dog 5 (Table I) was started on 20 mg. per kilogram and within 2 months he became cachectic, developed a normocytic anemia with 3.8 Gm. hemoglobin, and died.

Dog 6 (Table I) was started on 25 mg. hydralazine per kilogram body weight. Within 1 week she refused the medication and by 2 weeks she became so violent that it was even difficult to inject the drug. The animal was sacrificed 5 weeks after starting treatment, at which time her hemoglobin was 10.5 Gm.

Dog 7 (Table I) received 35 mg. per kilogram. Within one week she refused the hydralazine and had to be forcefed and then given parenteral material. She died 13 days after starting the drug.

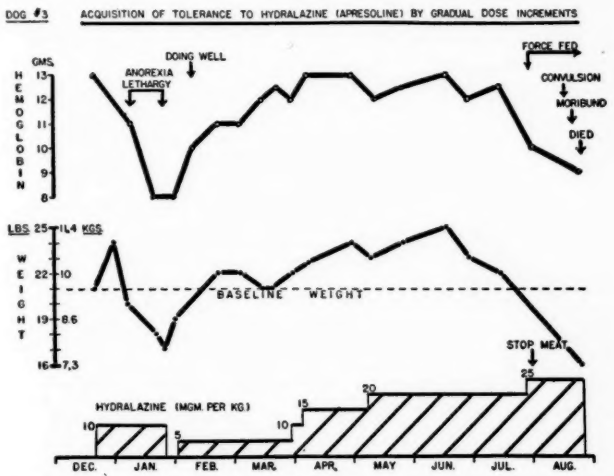

Fig. 1.—Course of Dog 3. Acquisition of tolerance to hydralazine (Apresoline) by gradual dose increments.

Dog 8 (Table I) received 50 mg. per kilogram. Two days after starting this dosage she salivated profusely and stood rigidly against the wall of her cage. She died 6 days after starting hydralazine in this dosage.

Most of the dogs were febrile during the latter part of hydralazine feeding. There was no evidence of arthritis at any time. (See addendum.)

LABORATORY STUDIES DURING THE COURSE OF HYDRALAZINE TREATMENT

Numerous L.E. cell preparations were performed by 5 different methods as outlined above and recorded in detail in the accompanying tables. At no time were L.E. cells seen by any of the methods. A few tart cells were noted in Dog 1. (Fig. 2). These are sometimes confused with true L.E. cells.

The anemia which appeared was normocytic. Attempts at performing a reticulocyte count failed because of inability of the dog's cells to stain in the usual manner with cresyl violet. Normoblasts were found in the blood

film of Dogs 3 and 5. Quantitative serum bilirubin studies in Dog 6 before and after hydralazine administration were both normal despite the fact that his hemoglobin dropped from 15 to 10.5 Gm. in 5 weeks while receiving 25 mg. per kilogram. The indirect bilirubin rose from 0.10 to 0.25 mg. per cent but little significance can be placed on this change. The gross autopsy findings of Dogs 7 and 8 which received 35 and 50 mg. per kilogram, respectively, showed bile staining of the tissues. Unfortunately, in these animals no bilirubin determinations were made. It is probable that the mechanism of the anemia is hemolytic. Further confirmation of this was shown by the microscopic studies presented below.

Leukocytosis occurred in all the dogs. There was not one instance of leukopenia. The sedimentation rate tended to rise with progressive toxicity.

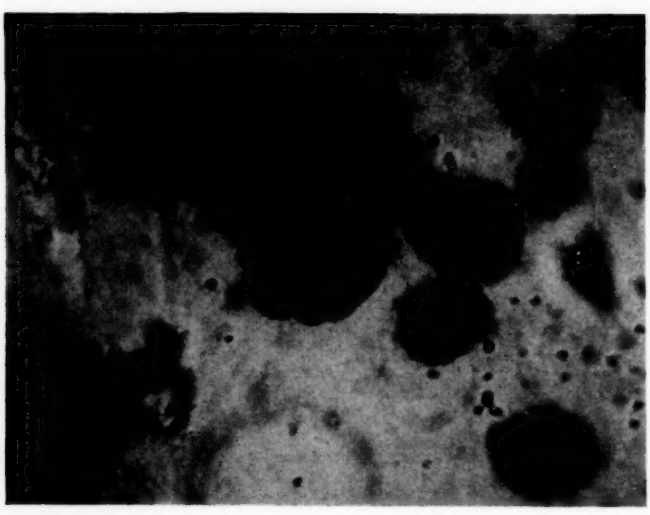

Fig. 2.—Tart cell from L.E. preparation of Dog 1. (×1,500; reduced ¼.)

AUTOPSY FINDINGS

Four dogs died and autopsy examination was performed 5 hours post mortem on 2 and 30 hours post mortem on the other 2. Three additional animals were sacrificed by Nembutal anesthesia when obviously moribund so that fresh tissues could be obtained. The only significant gross findings were noted in the two animals who received the largest amounts of hydralazine, namely, 35 and 50 mg. per kilogram. Their viscera were bile stained. The former animal in addition had an area 2 cm. in diameter and 3 mm. deep of deeply bile stained material in the liver which appeared necrotic. The bowel wall of the other dog was hemorrhagic. The remainder of the organs including the kidneys were normal.

Microscopically the most significant changes were found in the liver and spleen. There was congestion of both organs with hemosiderosis most marked in the more severely anemic animals. No onion skin lesions of the splenic

arterioles were found. Examination of the kidneys revealed increased glomerular cellularity over the control pretreatment biopsies which were available in 3 dogs. Interstitial mononuclear infiltration was noted in 6 of the 7 autopsied dogs. The infiltrate was most prominent in the subepithelial tissue of the renal pelvis and often extended along the course of the tubules. These lesions resembled low grade early human pyelonephritis. Both male and female dogs showed these changes. The females had never been catheterized; however, their urine specimens were obtained by sterile transabdominal puncture of the bladder. The urine of one male showed bacilluria prior to therapy. Two other dogs had bacilluria and pyuria during treatment. Sections of kidneys from 5 other normal dogs did not show this lesion. There was no evidence of wire loop lesions. No hematoxylin bodies were seen. Brain sections revealed generalized cerebral edema in 3 out of 5 dogs and congestion of the blood vessels in 2. Numerous cortical hemorrhages were noted in Dog 9 who died in status epilepticus. The remainder of the microscopic tissue was not remarkable except for pulmonary edema in several dogs. The myocardium was normal. Bone marrow sections were available in one animal (Dog 9). Marked hyperplasia was evident in the vertebral and rib specimens.

DISCUSSION

Hydralazine in doses of 20 mg. per kilogram daily produced a progressive toxic anemia, probably hemolytic in type, terminating in death by cachexia or convulsive seizures. These findings are similar to those of Earl.[5] Hydralazine is chemically related to phenylhydrazine, a drug which is known to cause hemolytic anemia. There has been no evidence in our studies that any changes even suggestive of systemic lupus erythematosus can be produced. How can one then explain the "hydralazine syndrome" in human beings? It has been shown that hydralazine is a strong skin sensitizing agent.[11] The febrile hydralazine syndrome occurs in about 8 to 10 per cent of patients receiving this drug.[1, 2] The incidence of a clinical picture suggestive of systemic lupus erythematosus with the findings of L.E. cells varied from 1.4 to 3.0 per cent in two large series of cases.[1, 2] Since the drug is a potent sensitizing agent and when injected intradermally produces delayed sensitization it is quite likely that the arthralgia and other early symptoms noted from this chemical are a serum sickness type reaction. One is led to wonder whether those patients who have such a reaction with the finding of true L.E. cells may not have latent lupus with a superimposed serum-sicknesslike reaction. The propensity of lupus patients to drug sensitization is well known. Many patients with systemic lupus erythematosus present themselves with hypertension, occasionally in the malignant phase. In our series of 220 cases of SLE we have seen several whose presenting problem was malignant hypertension. Two even received hydralazine for several weeks without any ill effects.

It seems likely, therefore, that we are dealing with 2 types of "hydralazine reactions," the most common being a drug sensitivity in a patient with essential hypertension and the other type being a patient with latent SLE whose

underlying disease is exacerbated by the use of hydralazine. In this latter type, the picture is typical of the underlying disease and L.E. cells are found. The disappearance of the symptoms and laboratory changes after withdrawal of a noxious agent SLE is common.

SUMMARY

Ten dogs have received hydralazine in dosages varying from 5 to 50 mg. per kilogram for as long as 12.5 months. The animals tolerated 10 mg. per day or less without difficulty and by gradually increasing the dosages tolerance can be developed to as much as 25 mg. per kilogram. The pattern and rate of toxic reactions varies directly with the dosage schedule. Utilizing 50 mg. per kilogram the animal died within a week whereas on 15 mg. per kilogram it required 3½ weeks or more. A progressive normocytic anemia, fever, anorexia, and cachexia appeared often with terminal convulsions. Leukopenia never appeared despite numerous tests. L.E. cells were not found at any time. Five different techniques were used for making the L.E. preparations. Autopsy studies showed congestion of the liver and spleen with hemosiderosis of both compatible with hemolytic anemia. Generalized cerebral edema was present. There were no renal or other changes suggestive of SLE.

ADDENDUM

Since listing the data in this paper three and one-half months have passed. Dogs 2, 4, and 10 are all alive and well on a maintenance dose of 20 mg. of hydralazine per kilogram per day. They have gained an average of one-half kilogram in weight during this period. Dog 2 has received the medication for a total of 16 months, Dog 4 for 12.5 months, and Dog 10 for 9 months. All their laboratory studies enumerated in the text, including sedimentation rate and hematocrit, are normal; L.E. cell preparations are all negative.

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CHEMICAL EVIDENCE OF TOLERANCE TO PHENOBARBITAL

IRVING SUNSHINE, PH.D.
CLEVELAND, OHIO

PHENOBARBITAL has been used therapeutically for many years. Its effectiveness as a sedative has long been recognized. In addition, the anticonvulsive properties of phenobarbital make it a major therapeutic agent in the symptomatic treatment of grand mal and status epilepticus. Thus, many patients are introduced to routine use of this drug. As a result of this prolonged therapy, habituation is not an infrequent occurrence. This may result in chronic intoxication and has been described clinically by Isbell.[1] Some individuals develop what seems to be a tolerance to this intoxication, and although this has been described clinically,[2-5] little chemical evidence has been offered to support it. This paper is a study of 8 cases of phenobarbital intoxication. The concurrent clinical and chemical data substantiate the thesis that tolerance does develop.

MATERIAL AND METHODS

Patients in various hospitals in Cuyahoga County (Cleveland, Ohio) who were hospitalized for acute barbiturate intoxication were studied. In some of these cases, atypical chemical data suggested tolerance to the drug in these individuals. In addition, several phenobarbital habituated epileptics, admitted to the hospital for the purpose of reducing or adjusting their phenobarbital therapy, were studied. Periodic blood and urine samples were taken from these patients. Simultaneously, their clinical conditions were observed and objectively rated as follows.

Stage I: Awake, competent, and mildly sedated.

Stage II: Sedated, reflexes present, prefers sleep, answers questions when roused, does not cerebrate properly.

Stage III: Comatose, reflexes present.

Stage IV: Comatose, no reflexes obtainable.

Stage V: Comatose, no reflexes obtainable, and with circulatory and/or respiratory difficulty.

These stages do not have definite limits, but blend into each other. The deep tendon reflexes were used as the criteria for the presence of reflexes. Corneal, pupillary, and abdominal responses are subject to more variability in relation to the levels of anesthesia. Gag and cough reflexes return at about the same time as the deep tendon reflexes; response to pain returns later. The respiratory difficulty in Stage V is due to direct depression of the medullary respiratory center and is not due to the secondary pneumonia which often develops in these patients.

All chemical phenobarbital determinations were done using a minor modification of the ultraviolet spectrophotometric technique described by Goldbaum.[6] All chemical data

From the Departments of Pharmacology and Pathology, School of Medicine, Western Reserve University and the Cuyahoga County (Ohio) Coroner's Office.
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are reported in terms of sodium phenobarbital concentration. Duplicate determinations agree within 10 per cent of each other.

Whenever possible, timed urine samples were collected to determine the renal clearance and daily excretion of phenobarbital.

RESULTS

Data obtained in the 8 patients studied are summarized in Table I. The first 4 patients were known epileptics who were hospitalized for adjustment of their epilepsy therapy. Three were withdrawn from phenobarbital and put on Hibicon and Dilantin. The fourth was continued on phenobarbital therapy.

TABLE I. PHENOBARBITAL TOLERANCE DATA

CASE	DOSE (MG./DAY)	BLOOD PHENOBARBITAL LEVELS IN VARIOUS CLINICAL STAGES				
		1	2	3	4	5
Norm		3.5	4.4	6.5	10.0	
J. M.''	1600—0	7.1	9.1			
M. H.''	128—640	5.6				
N. L.''	640—0		10.0			
R. N.''	576—0	6.7	11.5			
J. W.''		7.5	10.3			
J. U.		7.6	8.2	11.7	13.4	13.4
L. B.		7.3	8.8	10.5	12.5	17.0
N. I.''		7.7	8.2	10.6		

These cases marked '' represent known epileptics. The figures under the various clinical stages (1 to 5) are the blood phenobarbital levels (mg. per 100 ml. blood) obtained as the patient was observed to enter the given clinical stage. The values opposite "Norm" represent the average blood phenobarbital concentration observed in patients hospitalized due to acute phenobarbital intoxication.

The 4 other patients were suicide attempts. Two of these were known epileptics. One other, L. B., had been on prolonged barbiturate therapy prior to this incident. In only one patient no previous history of barbiturate exposure was elicited. None of the epileptics were seriously ill at any time during their hospital stay. Two cases, L. B. and J. U., were critically ill on admission. For 5 and 7 days, respectively, constant but conservative therapy, such as that

TABLE II. BLOOD PHENOBARBITAL LEVEL WITH RESPECT TO PATIENTS' CLINICAL CONDITION

PATIENT	BLOOD BARBITURATE LEVELS (MG. PER 100 ML.) PHENOBARBITAL					
	STAGE	V	IV	III	II	I
B. S.					4.0	2.4
I. B.				3.8		3.3
C. H.					4.3	
M. A.					4.6	3.6
G. W.				7.7		
R. H.				6.1		
W. Z.			11.0			
D. W.				7.4		3.9
T. L.						2.0
A. Z.						1.2
P. P.		20.6	9.2			
S. S.			9.2	5.0	4.2	3.5
E. G.				6.1		
J. D.						2.2

These data were obtained from patients (other than those reported in Table I) who were admitted to local hospitals for acute phenobarbital intoxication. The figures under the various clinical stages (1 to 5) are the blood phenobarbital levels (mg. per 100 ml.) obtained as a patient was observed to enter a given clinical stage. These data were used to determine the values used opposite "Norm" in Table I.

described by Nillson[7] and Eckenhoff,[8] was maintained by the attending physicians. At the end of these periods, both these patients were in Stage II with blood barbiturate levels of 8.2 and 8.8 mg. per cent, respectively.

In all 8 patients the blood phenobarbital level was 1½ to 2 times higher than "average" for a given clinical state. The blood phenobarbital level at which these patients were able to mentate properly was approximately 7.5 mg. per cent. The "average" intoxicated patient with this blood phenobarbital level would probably be in Stage III, comatose, with normal reflexes. (Table II.)

Renal clearance of phenobarbital was studied in so far as clinical facilities allowed. The clearances were calculated in the usual way: $C = \frac{U \times V}{B}$. U was the urine phenobarbital concentration and V the rate of urine flow in milliliters per minute. The blood phenobarbital concentration (B) was not corrected for protein binding. Fig. 1 shows that renal clearance is a function of the rate of urine flow.

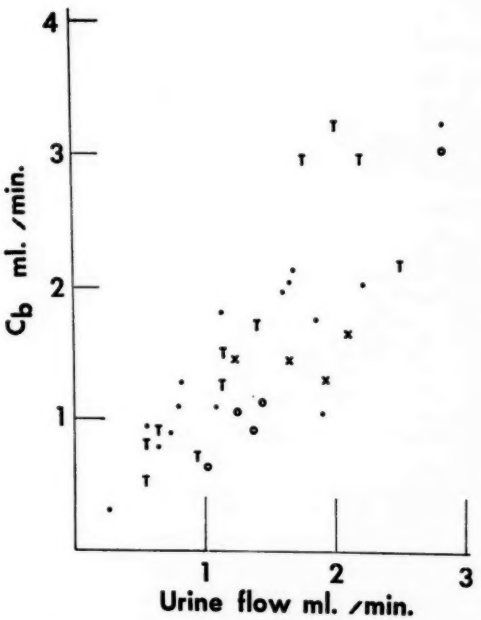

Fig. 1.—Renal clearance of phenobarbital vs. urine flow. Each symbol represents various renal clearances obtained from a given patient at various times during his hospital stay; 4 different patients were studied.

The data in Table III indicate the clearance is independent of the blood phenobarbital concentration. These data substantiate those reported by Lous.[9]

Urinary excretion of phenobarbital was also studied. The daily urinary excretion seemed to be independent of the dose. In 70 per cent of the analyses made, the daily phenobarbital excretion ranged from 80 to 160 mg. In most of the more serious phenobarbital intoxications, the average daily urinary excretion was 120 mg. The per cent of a given dose excreted daily is inversely proportional to the amount ingested (Fig. 2).

In 4 patients, the daily dose of phenobarbital was known. Whenever possible, 24-hour urine samples were collected from these patients to determine the percentage of the dose excreted each day (Table IV). These data substantiate the fact that the average daily phenobarbital excretion is about 30 per cent of the ingested dose. There have been similar statements in the literature,[10, 11] but some few investigators claim this value is as high as 60 to 80 per cent.

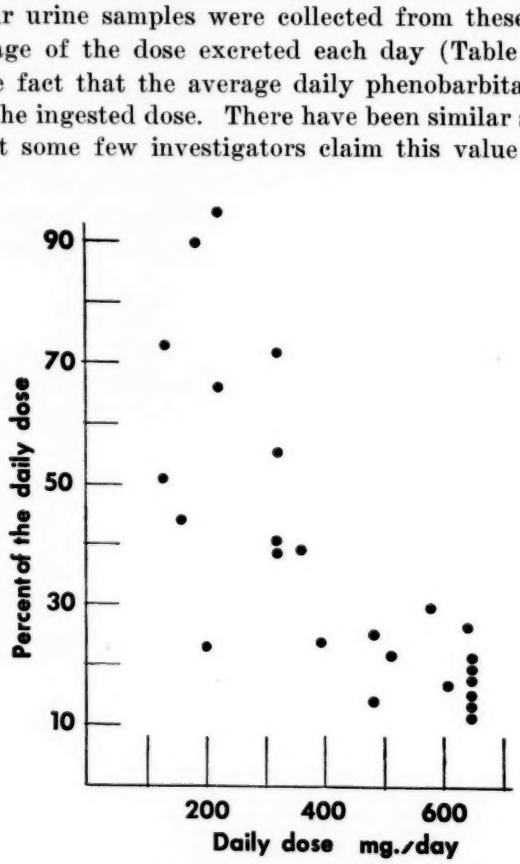

Fig. 2.—Urinary excretion of phenobarital.

TABLE III. RENAL CLEARANCE OF PHENOBARBITAL AS A FUNCTION OF BLOOD PHENOBARBITAL LEVELS

BLOOD LEVEL (MG. PER 100 ML.)	RENAL CLEARANCE (ML. PER MIN.)		
	RANGE	MEDIAN	CASES
2.0- 2.9	1.04, 1.46		2
3.0- 3.9	0.49-2.84	1.1	4
4.0- 4.9	0.73-3.1	1.3	14
5.0- 5.9	0.52; 0.80; 1.5		3
6.0- 6.9	1.2-2.2	1.7	5
7.0- 7.9	1.3-3.2	1.46	5
8.0- 8.9	0.78-3.08	1.2	8
9.0- 9.9	0.32-1.2	0.8	4
10.0-10.9	0.59, 0.83		2
11.0-11.9	0.94		1

TABLE IV. PER CENT OF DAILY PHENOBARBITAL DOSE* EXCRETED IN THE URINE

PER CENT EXCRETED	CASES
10-30	16
31-50	5
51-75	5
over 75	3

*This dose varied from 64 to 640 mg. per day.

DISCUSSION

The question of tolerance to barbiturates has been debated for many years. Much of the lack of agreement can be traced to several factors: (1) poor or loose definition of the terms tolerance and addiction; (2) differences in the time between doses, the size of doses, and the species and number of animals involved in the studies. Gruber and Keyser[12] have adequately reviewed existing animal studies on this topic. Their own definitive work indicated experimental animals showed a moderate degree of tolerance to the hypnotic action of various barbiturates. There was no increased tolerance to a lethal dose (LD 50), but some cross tolerance was demonstrated. The animals lost their acquired tolerance following cessation of barbiturate administration.

Idestrom,[13] working with previously undiagnosed epileptics, found that initial daily doses of phenobarbital impaired these patients' performances of standard tests. After prolonged therapy at the same dosage, this impairment was no longer apparent.

Isbell and Fraser[2] have described human tolerance to barbiturates clinically. They indicate this tolerance is never complete. Each individual builds his own tolerance to a certain level. Any increase in the barbiturate dose beyond this, even as little as 0.1 Gm., results in marked acute intoxication.

Wright[5] states that continuous therapy with barbiturates leads to tolerance, but that such tolerance is not great. In his study he only found 2 cases with levels comparable with those in Table I, while the rest of his cases show much lower levels. He also noted tolerance to the shorter acting barbiturates.

Lous[9] studied 20 epileptic patients on daily phenobarbital therapy. Of these, 70 per cent received 200 mg. or less daily. The remainder received no more than 400 mg. daily. He noted several of these patients were in a normal state of consciousness at a serum phenobarbital level of 5 mg. per cent. Many recent investigators also indicated that most patients are somnolent at this serum phenobarbital level.

The data presented in this study (Table I) corroborate the fact that tolerance to phenobarbital does exist. The 4 epileptics studied required considerably more phenobarbital to achieve a given clinical condition than does an "average" patient. For each clinical stage described, the blood phenobarbital levels, which are a direct function of the dose, were always much higher in the 8 patients studied than those found in other patients acutely intoxicated with phenobarbital (Table II).

Acute tolerance to large doses of Pentothal was reported by Brodie.[14] Lous[15] could not demonstrate this phenomenon with either barbital or phenobarbital. For acute tolerance, those patients with high initial doses should waken with higher blood barbiturate levels than those with lower initial doses. The data on the epileptic cases in this study do not indicate acute tolerance was achieved by any of these patients. Although the actual doses were not known in 2 cases, they obviously were not the same. The actual doses in the other cases are given in Table I. They vary considerably. However, the blood phenobarbital level for Stages I and II were reasonably similar in all the cases studied (Table I).

The renal clearances of phenobarbital and its urinary excretion are the highest generally obtained for any barbiturate, with the exception of barbital. Despite this fact, the absolute values are relatively small. Thus, excretion is only a small factor in the way the body handles phenobarbital. The largest portion of the drug is metabolized, probably by the liver. Although high urine flow gives a higher clearance, it is doubtful that this type of therapy is efficacious in a seriously intoxicated person. In these cases, the urinary excretion of 80 to 160 mg. daily is not significant. Much more heroic therapy is indicated in these acute episodes.[16, 17]

SUMMARY

1. Clinical and chemical tolerance to phenobarbital is demonstrated in 8 patients who have been on phenobarbital therapy for long periods of time. These patients recovered at much higher blood levels than "average" individuals.

2. The renal clearance of phenobarbital is directly related to the urinary flow rate.

3. The renal clearance values have no relationship to the blood phenobarbital concentration.

4. An average of 30 per cent of a daily phenobarbital dose is excreted by the kidneys.

5. In 70 per cent of the analyses made, the average urinary excretion of phenobarbital varied from 8 to 160 mg. per day.

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LABORATORY METHODS

DETECTION OF MYELOMA PROTEIN IN URINE BY A NEW QUICK METHOD

P. Efferøe, M.D., and B. Tidstrøm, M.D.
Copenhagen, Denmark

IN SOME patients suffering from myelomatosis the renal clearance of myeloma protein is so great in proportion to the rate of formation that the protein is not detectable in serum by electrophoresis or salting-out, owing to a very low serum concentration.

Electrophoretic demonstration of myeloma protein in urine is a relatively laborious process and consequently expensive, because prior to the electrophoresis the urinary protein has to be concentrated by salting-out and thereafter again isolated by dialysis.

Below we shall describe a simple method of detecting myeloma protein in urine, based on the differences in solubility at certain salt concentrations between myeloma protein and the protein occurring in urine from patients with nonmyelomatous renal diseases.

PRINCIPLE

In preliminary experiments we found that:

1. In urine pure myeloma protein is salted out as globulin (Fig. 1, *a*). The peak of the salting-out diagram varies from one patient to another, within a range of 30 to 60 per cent salt concentration using 3.6 mol potassium phosphate.

2. In urine nonmyeloma protein is salted out mainly as albumin. The peak of the salting-out diagram usually lies at about 75 to 80 per cent salt concentration. At 70 per cent salt concentration less than half of the total amount of protein had been precipitated in each of the cases tested (Fig. 1, *b*).

3. In cases of myelomatosis with myeloma protein in the urine the latter most often contains nonmyeloma protein as well (Fig. 1, *c*).

Whether in these cases more or less than half of *the total* protein content is salted out at 70 per cent salt concentration depends on the quantitative ratio between the myelomatous and the nonmyelomatous protein. At a low myeloma protein concentration and a high concentration of nonmyeloma protein less than half of *the total* protein may hypothetically have been salted out at a 70 per cent salt concentration.

The object of the present investigation has been to determine how often the presence of myeloma protein will cause precipitation of more than half of the *total protein* at a 70 per cent concentration of the stated salt solution, even if nonmyeloma protein is present at the same time. These preliminary results have

From III Medical Department of the Kommunehospital, Copenhagen, Denmark. Physician-in-Chief: Poul Iversen, M.D.
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been tested on 21 cases of myeloma proteinuria and the issue was compared with that of the simplest modification of the so-called Bence Jones test, the temperature flocculation test.

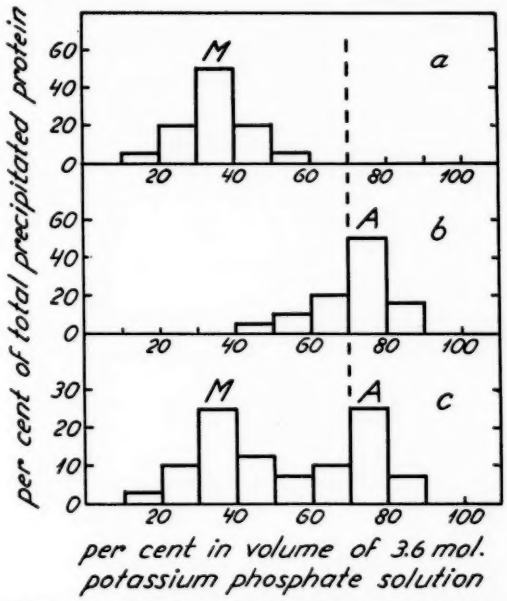

Fig. 1.—Salting-out diagrams for urinary protein (outlined). *a*, Myeloma protein. *b*, Nonmyeloma protein. *c*, Both forms of protein in the same urine. (100 per cent salt saturation = 3.6 molar equimolar monohydrophosphate and dihydrophosphate.)

MATERIAL

1. Of 21 patients with myeloma proteinuria,* the diagnosis in 18 had been made from the finding of myeloma cells in the bone marrow.† During the development of the method, the remaining 3 were disclosed on routine examination of all the patients with proteinuria in the department. In 2 of these the diagnosis was verified by bone marrow analysis and in all 3 cases by electrophoretic analysis of the urinary protein.

Urine specimens from these 21 patients were submitted to a salting-out test as well as a temperature flocculation test. In the cases where one of these two tests was negative, electrophoretic analysis of the urinary protein was carried out as well.‡

2. The urine of 50 consecutive patients was proved by Heller's test to contain protein owing to acute or chronic renal diseases of nonmyelomatous nature. Urine specimens from these patients were submitted to a salting-out test also.

3. The urine of 21 unselected patients contained protein, as revealed by Heller's test, due to acute or chronic renal diseases of a nonmyelomatous nature. Urine specimens from these patients were submitted to a temperature flocculation test.

REAGENTS

1. *Salting-out Test.—*

3.6 molar equimolar mono- and dihydropotassium phosphate solution:

245.0 Gm. KH_2PO_4 (analytic salt)

313.6 Gm. K_2HPO_4 ("rein" quality, Riedel de Haën).

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*We thank the Chiefs of the respective departments who have sent us urine from myelomatous patients.

†We are greatly indebted to Dr. H. Gormsen of the Department of Forensic Medicine, University of Copenhagen, and Dr. Charles Johansen, Prosector at the Øresundshospital, Copenhagen, for information on the myeloma patients.

‡The electrophoretic analyses were carried out by Dr. Niels Harboe, the electrophoretic Laboratory (Tiselius apparatus), Department of General Pathology, University of Copenhagen.

The salts are dissolved in a 1000 ml. flask in distilled water up to the mark. The opalescent solution is filtered clear through hard filter paper (e.g., Schleicher & Schüll 602 eh.).

Control of the solution: A correct proportion between mono- and dihydrophosphate and a correct total salt concentration are secured by means of pH measurement and determination of specific gravity.

In the dilution of 1 ml. of salt solution to 35 ml. of distilled water the pH has been found to be 6.80 ± 0.01. The relative specific gravity against water at the same temperature has been determined at 1.376 ± 0.003.

To obtain precipitation of all the urinary protein it was necessary to work with highly concentrated salt solutions. The salt solution used is at room temperature concentrated to almost 100 per cent saturation, and the dissolution of the salts may require standing for some time and powerful shaking. Higher temperatures will increase the solubility. Since, however, it seemed in a few experiments as if the salting-out capacity increased if the solution had been heated, we chose to keep the temperature within 30° C. while dissolving the salts.

Monohydropotassium phosphate is noncrystallizable, being, therefore, unobtainable in pure form. The stated control of the solutions was accordingly necessary. Monohydrosodium phosphate, as used by S. P. L. Sørensen, is obtainable in pure crystalline form. It is, however, less readily soluble than monohydropotassium phosphate and the salt concentration at 100 per cent saturation will be so low that not all the protein in the urine can be precipitated.

The specific gravity was determined by weighing the salt solution and the distilled water in the same 20 ml. flask.

2. *Temperature Flocculation Test.*—As buffer we used the following solution:

0.07 M NaH_2PO_4
0.01 M Na_2HPO_4
pH 6.0 at ionic strength 0.1 of the solution.

PROCEDURE

1. *Salting-out Test.*—The test requires 3 ml. of clear filtered urine. The solutions are measured off into approximately uniformly calibrated test tubes, as shown in Table I, and the tubes are inverted cautiously 4 times. A positive result for myeloma protein is indicated by a more extensive precipitation in tube No. 1 than in tube No. 2. A negative result does not exclude myeloma protein (see results). If in doubt as to which of the tubes presents the most extensive precipitation, we may submit the specimens to photometry.

TABLE I. SALTING-OUT TEST

TUBE NO.	POTASSIUM PHOSPHATE SOLUTION (ML.)	H_2O (ML.)	URINE (ML.)	TOTAL VOLUME (ML.)	SALT-CONCENTRATION (PER CENT SATURATION)	CONCENTRATION OF URINE (PER CENT)
1	14	4	2	20	70	10
2	18	1	1	20	90	5

The test is inapplicable for urine that cannot be filtered clear, as well as for urine to which chemicals have been added, e.g., owing to bladder lavage.

The mixture of urine, water, and salt solution should be inverted only 4 times and should not be shaken, as shaking will cause an inconstant increase of the turbidity.

Readings can be taken immediately, and must be taken within half an hour of mixing, because standing will also cause an inconstant increase of the turbidity. For the photometry we used Pulfric's step-photometer with filter No. 6 and a light path length from 0.5 to 5.0 cm., dependent on the degree of turbidity. In the present investigations the naked-eye readings were in all cases controlled by photometric readings.

2. *Temperature Flocculation Test.*—Equal parts of the buffer and filtered clear urine, usually 2 ml. of each, are heated slowly under stirring on water bath, with a thermometer dipped in the mixture.

Flocculation at 65° C. or lower temperature is reckoned as positive for myeloma protein. A negative result does not exclude myeloma protein (see results).

To avoid a too rapid rise in temperature when the critical temperature of 65° C. was passed we always removed the source of heat as soon as the thermometer showed 55° C. and thereafter continued with very slow intermittent heating.

EXPERIMENTAL

The salting-out test was positive in 19 of the 21 patients with myeloma proteinuria (Table II). In the 18 cases the positive reaction was directly visible by the naked eye and confirmed by photometry. In the nineteenth the reaction was doubtful when viewed directly, but photometry showed it to be positive.

In the 7 cases with a positive salting-out test, but a negative temperature flocculation test, the urinary protein was submitted to electrophoretic analysis which revealed myeloma protein in the urine in all cases.

In one of the two patients suffering from myelomatosis with a negative salting-out test the temperature flocculation test was positive. Repeated salting-out test on urine produced a few weeks later gave a positive result. As, however, the first test was negative, possibly due to an error of the routine performance, it was set out as negative in Table II.

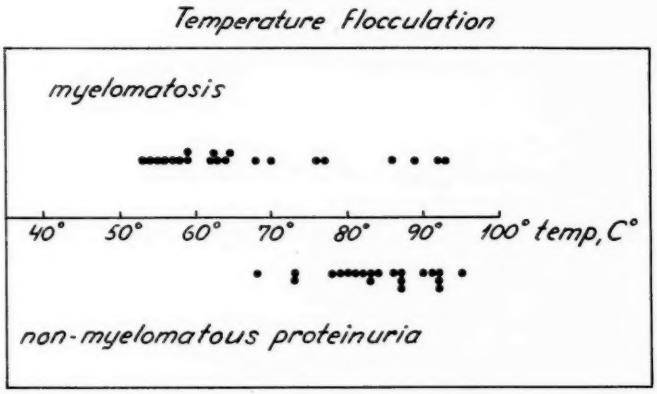

Fig. 2.—Flocculation temperature for urinary protein at pH 6 in patients with myelomatosis and other forms of proteinuria.

In all 50 patients with renal diseases (series 2), the salting-out test gave negative results when viewed directly (confirmed by photometry). While the protein present in urine from the patients of series 3 with renal diseases in no instance flocculated at temperatures under 65° C., a lower flocculation temperature was seen in urine from more than half of the patients with myelomatosis (Fig. 2).

TABLE II. FREQUENCY OF POSITIVE REACTIONS IN MYELOMA PROTEINURIA

NUMBER	SALTING OUT TEST	TEMP.FLOCCULATION TEST "BENCE JONES"	ELECTROPHORESIS
12	Positive	Positive	Not tested
7	Positive	Negative	Positive
1	Negative	Positive	Positive
1	Negative	Negative	Positive
21	19	13	

After 3 weeks, repeated testing of urine from the patient set out in the table as negative to the salting-out test and positive to the temperature flocculation test gave positive reaction to the salting-out test (see text).

If we regard a flocculation temperature under 65° C. as positive with a view to myeloma protein, this reaction was positive in 13 of the 21 patients with myeloma proteinuria (Table II).

DISCUSSION

The properties of the protein described by Bence Jones[1] (1848), in his only case reported of myeloma proteinuria were, from a modern point of view, rather inaccurately explained. He only stated that by adding acid the urine became turbid, and that the turbidity disappeared by boiling and returned after cooling down. The numerous subsequent Bence Jones tests, often modified beyond recognition, work with highly different pH values and flocculation temperatures. The modifications, e.g., that of Jacobson and Wilner,[2] aimed at verifying the properties demonstrated by Bence Jones in the presence of other protein as well, such being often found, are laborious and the chance of a falsely negative result is great. They must therefore be regarded as obsolete since the introduction of the technique of electrophoresis.

The modifications based on the fact that certain myeloma proteins have a fairly low flocculation temperature are also called Bence Jones tests by some workers, though they have nothing to do with Bence Jones' original description of the properties of myeloma protein.

The temperature flocculation test, just as the salting-out test, has the great practical advantage of being easily carried out, especially compared with electrophoretic analysis of urinary protein.

The salting-out test and the temperature flocculation test both require only one reagent. For the untrained person, the salting-out test is easier to read and quicker than the temperature flocculation test, and positive findings in myeloma proteinuria are considerably more frequent, in the present series being about 90 per cent against about 60 per cent.

In our experience, a salting-out test, and possibly also a temperature flocculation test, ought to be carried out in all cases of proteinuria where a nonmyelomatous renal disease is not unquestionably present. In cases of positive results the diagnosis should be verified by electrophoretic analysis of the urinary protein.

The fact that in a medical department we disclosed within one year 3 cases of myeloma proteinuria by means of the salting-out test illustrates the value of this test.

SUMMARY

Myeloma protein can be detected in urine by a simple method based on the difference in solubility in certain salt solutions between myeloma protein and protein occurring in the urine of patients with nonmyelomatous diseases.

Among 21 patients with myeloma proteinuria the simplest modification of the "Bence Jones test," the temperature flocculation test, was found positive in about 60 per cent, whereas the salting-out test was positive in about 90 per cent. Falsely positive reactions were not observed in cases of nonmyelomatous proteinuria.

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A NEW METHOD FOR THE ISOLATION OF TUBERCLE BACILLI FROM FECAL SPECIMENS

LEWIS J. GRIFFITH, PH.D., AND SALVATORE A. DENARO, M.S.
BATAVIA, N. Y.

INTRODUCTION

THE time-proved method for the isolation of tubercle bacilli employs either a sputum or gastric specimen. Before the advent of chemotherapy, this method was adequate. However, very early in the use of antituberculous drugs,[1] sputum reduction was observed to be so pronounced as to approach the disappearing point, making gastric lavage necessary. With some patients, this presents no problem. In other patients, it is a major undertaking to collect a gastric washing. It is desirable that an alternative method be available by which cultures may be obtained.

The use of an improved digestion method[2] and a flotation process suggested by Mudd and Mudd,[3] in 1924, have made it possible to use fecal specimens for culture of tubercle bacilli successfully. The purpose of this work was to evaluate a trisodium phosphate method for decontamination and a mineral oil method for concentration of the tubercle bacilli from fecal specimens.

MATERIALS AND METHODS

Only patients giving positive sputum cultures by standard methods were included in this study. A 2-3 Gm. fresh fecal specimen was added to a screw cap test tube containing 15 ml. of saturated aqueous trisodium phosphate solution. The specimen was shaken in a paint shaker for 15 minutes to obtain complete emulsion prior to incubation at 37° C. for 12 hours. Flotation of the tubercle bacilli was accomplished by the addition of 4 ml. of neutral mineral oil with an additional 5-minute shaking. The oil layer was allowed to separate out and cultures were made using only the oil layer. Three tubes of Löwenstein's medium (Jensen modification) were seeded for each specimen. Weekly readings were taken on the tubes for eight weeks. The amount of contamination was noted and the time of first appearance of tubercle bacilli on cultures recorded.

RESULTS

In all, 102 specimens were processed in the manner described above. The decontamination was very satisfactory in that only two specimens of the total were lost because of contamination. A total of 26 cultures produced growth that was typical of *Mycobacterium tuberculosis*. A representative number of these acid-fast cultures were injected into guinea pigs and were found to be virulent. No particular differences in colony form or rate of growth were observed in the fecal isolations as compared to colonies from sputum or gastric specimens.

From the Veterans Administration Hospital, Batavia, N. Y.
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DISCUSSION

The isolation of *Myco. tuberculosis* in 25 per cent of the fecal specimens makes this method usable under certain circumstances. Our experimental data agrees with that obtained by Petroff in 1915[4]—to isolate tubercle bacilli directly from feces is not easily accomplished. A thorough decontamination is the most important step in isolating tubercle bacilli from fecal specimens.

Since our method does not recover the tubercle bacilli in a number of the cases, it is not a substitute for the culturing of sputum or gastric specimens. However, in cases where no sputum is present and gastric lavage cannot be tolerated, fecal culture may prove of considerable use. Enzymatic destruction of the tubercle bacilli in the intestinal tract was not considered as a factor in this work. The use of a purged specimen and immediate pH adjustment to inactivate the proteolytic enzymes might be an important factor in improving this culture method.

SUMMARY

A trisodium phosphate digestion and mineral oil flotation method for recovering tubercle bacilli from fecal specimens has been described. Some methods for improving the culture recoveries are discussed.

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AN EVALUATION OF THE SAGI-PLESS CANCER DETECTION TEST

THOMAS F. SELLERS, JR., M.D.
ATLANTA, GA.

IN RECENT years considerable effort has been devoted to the development of a simple screening test for the presence of malignancy. Numerous procedures have been proposed from time to time, measuring various enzymatic, immunologic, biochemical, or physical properties of blood or serum.[1-5] The enthusiastic initial reports have been followed, almost without exception, by later evaluations which have revealed such a high incidence of false positive and false negative results that the test has been discarded.[6-12]

Sagi and Pless[13] have recently described the preliminary results with a technique of differentiation of sera on phenol. These authors observed a very low incidence of false positive tests (2 of 1,500 or 0.13 per cent) and a larger but acceptable incidence of false negative tests (30 of 357 or 8.4 per cent).

The mechanism of this reaction is thought to be an interplay of the precipitating effect of diluted aqueous phenol, and the dissolving effect of concentrated phenol, upon proteins. Variations in the observed reaction are probably dependent upon qualitative and possibly quantitative differences in the serum proteins. That these differences may be specific for the presence or absence of malignant neoplasms is the basis for the use of this reaction as a screening test for cancer.

This report describes the results of an evaluation of the Sagi-Pless test at a 300-bed Veterans Administration Hospital.

MATERIALS AND METHODS

Tests were performed on 101 persons, all of whom were patients in the hospital. Initial screening of the patients excluded those having certain nonmalignant diseases described by Sagi and Pless[13] as causing false positive results, i.e., uncontrolled diabetes, pernicious anemia, renal disease with nitrogen retention, viral hepatitis, and advanced cirrhosis. No patients with these diagnoses were included except 5 with mild cirrhosis (one had a weakly positive reaction and 4 had negative reactions).

In addition, Sagi and Pless noted that false positive reactions were sometimes observed in patients having recently received certain agents, such as blood transfusions, intravenous fluids, steroid hormones, anticoagulants, antibiotics, and salicylates. The widespread use of these agents made it impossible to avoid including a few such patients although an effort was made to exclude them. Radiation therapy and excisional surgery were listed as possible causes for reversion of a positive test to negative. Treatment with any of these drugs or procedures was carefully noted and the results considered separately.

From the Medical Service and Research Laboratories, Veterans Administration Hospital, Atlanta, Ga., and the Department of Medicine, Emory University School of Medicine.
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A diagnosis of malignancy was based in every case upon histologic examination of biopsy or post-mortem tissues. Those suspected of malignancy but in whom no pathologic confirmation was available were also listed separately.

The procedure used was as originally described[13] and was followed without alteration. Fasting venous blood specimens were collected, the sera separated and incubated in a water bath at 33° C. for 4 hours. Hemolyzed or lipemic specimens were not used. A measured amount (0.12 ml.) of a phenol-glycerine mixture of pH 8.0 was pipetted into each cavity of a conventional agglutination slide. Serum was then drawn up into a 1.0 ml. pipette fitted with an 18-gauge hypodermic needle with the point ground off smoothly. One drop of serum was allowed to drop from the needle tip onto the phenol pool. The distance of fall was carefully maintained at 6.0 mm. by means of a clear plastic table with small holes bored therein which allowed the needle to rest on its hub while the tip was exactly 6.0 mm. from the surface of the phenol. The reaction of the serum drop was observed. A drop which coagulated into a spherical white plug and slowly dissolved as it floated on the surface was read as negative. Complete dissolution in these instances required fully 20 to 30 minutes. A drop which rapidly disintegrated over the surface, dissolving immediately or within a few minutes, was read as positive.

The differentiation between the positive and negative reactions for a given serum drop was unequivocal in every instance. Three slide cavities were used for each specimen simultaneously. In several instances, only one or two of the triplicate tests were positive while the others were negative. This variable response with replicate determinations was confirmed by repeated testing of the same specimen. Such results were recorded as weakly positive and were considered separately. All tests were performed and read by the same individual. The results were recorded by serial number without reference to the patient's name or clinical diagnosis.

RESULTS

Of the 101 patients included in this series, 28 gave a positive test, 59 gave a negative test, and 14 were considered weakly positive. Table I indicates a breakdown by type of diagnosis of those tested. Of the 28 positive tests, 10 or 36 per cent were in patients without malignancy or question of malignancy and who had not received drug therapy known to cause false positive reactions. If those with weakly positive reactions are included, 16 of 42 or 38 per cent were in the false positive group. Table I also includes a similar analysis of those with a negative test. Of these, 6 of 59 or 10 per cent were in patients with diagnosed malignancy without recent treatment which might have reverted a positive test to negative. Of these 6, 3 had evident metastatic disease; the other 3 had Hodgkin's disease, bronchogenic carcinoma, and neurofibrosarcoma, respectively.

When considering the series by diagnosis, 63 patients had benign conditions and 23 had malignant ones. Eight were suspected of harboring malignancy which was not demonstrable and 7 were considered to have benign conditions although possibly malignant. These included 5 with gastric ulcer, benign by clinical criteria but without surgical or pathologic confirmation. Considering only those in whom the diagnosis was unequivocal, Table II shows that among the benign cases there were 40 with a negative test. In addition, a positive test was obtained in 7 patients who had received certain drugs which might have caused a false positive result. Assuming that all of the latter were falsely positive because of drugs, then 47 of the 63 or 75.8 per cent

showed the correlation of a benign diagnosis with a negative test. Eleven or 15.9 per cent were unexplained false positives, or if the weakly positive results were included, the false positive percentage was 25.4.

There were 23 patients with diagnoses of malignancies with the degree of involvement ranging from minor skin malignancy in one case (basal cell carcinoma) to generalized metastases from renal, gastric, bronchial, or colonic carcinomas (Table III). With few exceptions, the patients were hospitalized as a direct result of their malignant process which was usually far-advanced. Table II also includes a breakdown of the diagnoses of malignancy. There were 6 false negatives or 26.1 per cent of the total number with malignancy. Thirteen of the 23 or 56.5 per cent showed the correlation of a positive test with the presence of malignancy. An additional 4 or 17.4 per cent were negative reactions that may be explained by preceding therapy.

Although no effort at systematic retesting was made, 9 patients had duplicate tests. Two of these were positive in one instance and negative in another. No explanation for this was apparent; both patients had benign conditions.

TABLE I. RELATION OF DIAGNOSIS TO SAGI-PLESS REACTION

TYPE DIAGNOSIS	POSITIVE	WEAKLY POSITIVE	TOTAL POSITIVE	NEGATIVE
Malignancy proved	7	5	12	6
Malignancy suspected unproved	4	1	5	3
No malignancy suspected	10	6	16	40
Probably benign but malignancy a possibility	1	1	2	5
No malignancy suspected: patient had received certain drugs	6	1	7	–
Malignancy proved: patient had recent x-ray therapy	–	–	–	5
	28	14	42	59

TABLE II. SAGI-PLESS REACTION IN PATIENTS HAVING UNEQUIVOCAL DIAGNOSES

TYPE REACTION	DIAGNOSIS OF BENIGN CONDITION	DIAGNOSIS OF MALIGNANT NEOPLASM
Negative	40	6
Positive	11	5
Weakly positive	5	4
Positive: result possibly related to treatment	7	4
Negative: result possibly related to treatment	0	4
	63	23

DISCUSSION

It is obviously inaccurate to consider the group which received drugs as all drug-induced false positive in one instance and all genuinely positive in the next. This tends to weigh the figures in favor of the accuracy of the test. This was purposely done, however, in order to present the procedure to the best advantage, since even then it does not appear to qualify as an accurate screening or diagnostic test for cancer. Mackenzie,[14] in the discussion of the Sagi-Pless paper, mentions three series utilizing the phenol-serum reaction

TABLE III. RELATION OF TYPE OF MALIGNANCY TO SAGI-PLESS REACTION

TYPE MALIGNANCY	POSITIVE	WEAKLY POSITIVE	NEGATIVE
Bronchogenic carcinoma	3*		3*
Hodgkin's disease	1	1	2
Adenocarcinoma stomach	1		1*
Lymphatic leukemia, chronic	1	1	
Basal cell carcinoma		1	
Adenocarcinoma, colon			1*
Hypernephroma			1*
Lympho-epithelioma			1
Multiple myeloma		1	
Myelogenous leukemia, chronic			1
Neurofibrosarcoma			1
Squamous cell carcinoma, mouth		1*	
Undifferentiated cell carcinoma	1*		
	7	5	11

*Metastatic disease present.

with an accuracy ranging from 92 to 96 per cent. This high level of correlation was not achieved in this series, which was able to predict the presence or absence of malignancy in only 61.6 per cent. Dunn and Greenhouse,[15] whose criteria for an acceptable screening test for malignancy are widely accepted, indicate that such a procedure should be positive in at least 90 per cent of malignant cases and should be negative in no less than 95 per cent of non-malignant conditions.

It should be pointed out again that this series was composed entirely of hospitalized patients. Many of those with cancer had far-advanced disease. Since no outpatient examinations were included, very few cases of early malignancy were evaluated. Without long-term follow-up, it is not possible to say whether any of the presumed false positive group will turn out to have had early, undetected malignancy at the time of testing. However, each had the benefit of a complete hospital examination without evidence of malignancy being found. In most instances, no further examinations would have been performed without a lapse of time, even if a higher index of suspicion had resulted from the positive test. The relative absence of early malignancy may account for some of the differences noted between this study and the Sagi-Pless series, although as Homburger[9] has pointed out, it is natural to assume that a test for malignancy would show a greater degree of positive correlation in far-advanced cases.

SUMMARY

The results of an evaluation of the Sagi-Pless cancer detection test are presented. After allowing for recognizable causes of false positive and negative reactions with this test, there were 26.1 per cent false negatives and 25.4 per cent false positives. These figures greatly exceed the 10 per cent false negative and 5 per cent false positive reactions considered to be the maximum allowable for a practical screening test for cancer.

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A QUANTITATIVE MICROMETHOD FOR THE ISOLATION AND LIQUID SCINTILLATION ASSAY OF RADIOACTIVE FREE AND ESTER CHOLESTEROL

JON J. KABARA, M.S.
CHICAGO, ILL.

INTRODUCTION

FOR effective utilization of radioisotopes in biologic and medical research, analytic methods are needed that permit accurate quantitative analysis and radioassay on the same sample.[1] Ideally, the procedure should not require exorbitantly large samples and should yield sufficient material for duplicate or triplicate analyses. When radioassay is performed by means of a liquid scintillation counter, the purified sample must be in a suitable state to permit solution or suspension in the scintillator solvent system. The procedure for carbon^{-14} or tritium labeled cholesterol, which is described in this report, is a modification of earlier methods.[2, 3] It was made applicable to both blood and tissue samples and was adapted for use with a liquid scintillation counter. The present technique was used to determine the specific activities of cholesterol found in 0.5 to 1.0 ml. of human plasma after the injection of 100 to 200 μc of sodium acetate 1-C$^{4-6, 14}$ as well as the cholesterol isolated from most organs of the mouse after 1.0 μc of the same precursor. In the case of human plasma, the activity of the sample containing 0.250 mg. of free cholesterol may be as low as 10^{-4} μc/mg. of carbon^{-14} or 10^{-3} μc/mg. of tritium.

MATERIALS AND METHODS

The reagents used in these experiments are all C.P. or Reagent Grade Chemical and further purification was found unnecessary. The quality of dioxane used was critical; however, Eastman Kodak Co. was found to be a reliable source of commercial dioxane. A comparison between this commercial source and the freshly distilled dioxane did not warrant the use of the tedious purification procedure. The Packard Tri-Carb liquid scintillation counter Model 314 was used to count both carbon-14 and tritium samples separately or simultaneously.[7]

PROCEDURE

Extraction of Plasma Cholesterol.—Fifteen volumes of acetone-alcohol (1:1) were added to 1.0 ml. of plasma at room temperature. Heating on a steam bath was unnecessary[3] but it was important to *add the solvent to the plasma* in order to precipitate the protein into a finely divided state. The mixture was filtered through Whatman No. 1 filter paper, and the filtrate collected in conical-bottom centrifuge tubes. If the solution was not clear at this point, it was refiltered. To the clear filtrate, a few drops of 10 per cent acetic acid were added.

From the Argonne Cancer Research Hospital and Department of Pharmacology, The University of Chicago, Chicago, Ill.

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Extraction of Tissue Cholesterol.—Tissue samples of convenient size were rinsed in distilled water, blotted dry, and frozen in test tubes immersed in a mixture of dry ice and acetone. The weight of the frozen sample was recorded as "wet weight." The tissues (0.5 to 1.0 Gm.) were ground in 30 to 40 ml. of acetone-alcohol (1:1) solution until a fine suspension of the tissue protein was achieved. The mixture was filtered, the precipitated proteins were dried, and their weights recorded as "dry weight" (lipid-free).

Isolation of the Free Cholesterol.—The cholesterol filtrates, after evaporation to more convenient volumes (10 ml.), were ready for digitonin addition. On the basis of estimates of the amount of free cholesterol in either the plasma or the tissue filtrate, approximately four times this amount of digitonin as a 1 per cent solution in 50 per cent ethanol was added. Larger amounts of reagent were avoided.[8] The mixtures were then allowed to stand overnight at room temperature so that complete precipitation would occur. After the digitonide formed, the mixture was centrifuged at 2,000 r.p.m. for 10 to 15 minutes. The supernatant fluid was then carefully decanted and the precipitate washed thoroughly by a jet stream of acetone-alcohol (1:1) solution ejected from a syringe. The washed digitonide was recentrifuged and rewashed with acetone-ether (1:2) and finally with ether alone. The washings were all combined with the first filtrate and used to estimate ester specific activity.

Isolation of Ester Cholesterol.—The above precipitation with digitonin does not remove all of the Liebermann-Burchard positive material from solution. This was demonstrated by attempting to isolate purified free cholesterol (10 mg.) by adding 4.0 ml. of 1 per cent digitonin (50 per cent alcohol) to the acetone-alcohol (1:1) solution. The filtrates, after digitonide formation and separation, were evaporated to dryness and the amount of residue was determined by the Liebermann-Burchard reaction. The results of 13 determinations showed that about 3 to 5 per cent of digitonide was in the total washing residues, or a solubility of 0.6 to 1.0 mg./100 ml. Contamination due to the solubilized digitonide and/or uncombined free cholesterol need be considered *only* when ester fractions are isolated shortly after acetate injection—the reasons being that after a sufficient time, the two specific-activity curves of free and esterified cholesterol are "essentially" the same.[5, 9, 10] In those instances where the ester determinations were made, the combined washings as well as the filtrate from the "free" cholesterol were evaporated to dryness and extracted with 3 portions of hot petroleum ether (40 to 60° C.). The ether was then blown off gently with nitrogen and the ester fraction reconstituted to volume (10 to 20 ml.) with acetone-alcohol (1:1) solution. Hydrolysis of the ester was accomplished by adding 8 to 10 drops of potassium hydroxide (50 per cent) to the solution and then heating the tubes on a water bath at 40 to 50° C. for 45 minutes. The samples were then removed and allowed to cool to room temperature before acidifying to phenolphthalein with 10 per cent acetic acid. The steps after acidification are similar to those given for isolating "free" cholesterol.

Quantitative Measurement of the Digitonide.—In order to carry out the colorimetric analyses and radioactive assay on the same sample, a solvent was necessary that would dissolve the digitonide, give the Liebermann-Burchard (L-B) color, and still retain those properties suitable for acting as a solvent in liquid scintillation counter. A system possessing these properties was composed of dioxane and phosphoric acid (ortho) in a ratio of 99:1 (v/v). Various combinations of solvent systems as well as other acids with dioxane were tested and found to be unsatisfactory.

The dioxane was therefore added to the dry precipitate, and the sample tubes were placed in a boiling water bath until solution took place. Since the standard curve has an upper limit of 0.500 mg./ml., the amount of dioxane added to each sample tube depended upon the sample size. After the digitonides were dissolved, the solutions were cooled to room temperature and adjusted to a convenient known volume. A one-milliliter aliquot was taken for quantitative determination while the other part of the sample (usually 2 ml.) was used for assaying the amount of radioactivity.

The usual ratio of sulfuric acid:acetic anhydride (1:20) with dioxane had the disadvantage of slowness in color development as well as poor sensitivity. A ratio of 1:10 developed a color with cholesterol that was sufficiently stable and gave the desired sensitivity (Fig. 1). Because of the exothermic heat produced when adding this ratio of sulfuric acid to the anhydride, the acetic anhydride was cooled in an ice bath before and during the addition of the mineral acid. The mixed L-B reagent was then allowed to come to room temperature (25 to 27° C.) and was used within one-half hour after its preparation.

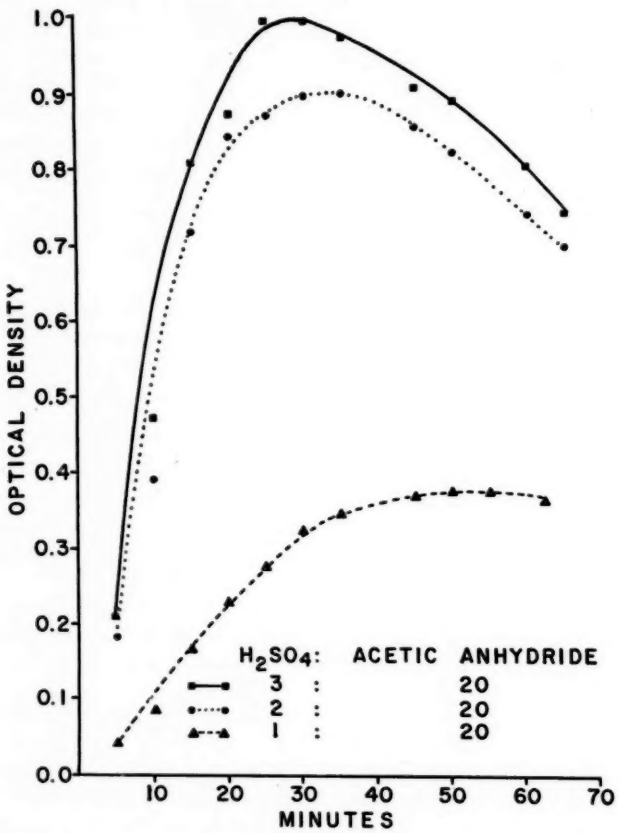

Fig. 1.—Kinetics of Liebermann-Burchard reaction: with cholesterol dissolved in dioxane, optimum operating ratio of sulfuric acid:acetic anhydride was found to be 2:20.

Standards containing various amounts of cholesterol (0.100 mg. to 0.500 mg.) dissolved in one milliliter of dioxane were prepared and mixed with 2.0 ml. of sulfuric acid-acetate anhydride mixture (1:10). As previously demonstrated,[11] *no special precautions concerning the exposure of the samples to light need be taken.* Because of the instability and slight variation in composition (acetic anhydride:sulfuric acid ratio) of the Liebermann-Burchard reagent from experiment to experiment, the maximum optical density rather than the O.D. at a particular time was used. The maximum optical density of the solutions was read at 620 mμ on a Beckman Model B Spectrophotometer. A special aluminum disk (2.4 mm. × 0.9 mm.) was machined which permitted the adaptation of the smaller volumes to this spectrophotometer. By the above procedure, a standard curve was drawn that was used for routine daily assays of cholesterol. Periodic monthly checks of the standard curve for over a year have failed to reveal any discrepancies.

TABLE I. EFFECT OF PHOSPHORIC ACID CONCENTRATION ON LIQUID SCINTILLATION COUNTING

H_3PO_4 IN DIOXANE (V/V) (PER CENT)	C.P.M.	C^{14} COUNTING EFFICIENCY (PER CENT)
0.0	567	47.1
0.5	548	45.5
1.0	503	41.8
2.0	501	41.6
4.0	463	38.5

Standard counting solution = dioxane:toluene:ETOH (5:10:1).

TABLE II. EFFECT OF DIOXANE CONCENTRATION ON LIQUID SCINTILLATION COUNTING

DIOXANE (1 PER CENT H_3PO_4) (ML.)	TOLUENE (ML.)	ETHANOL (ML.)	CARBON-14 (PER CENT EFFICIENCY)	TRITIUM (PER CENT EFFICIENCY)
0	14	1.0	47.3	3.84
2	12	1.0	45.8	3.53
4	10	1.0	44.3	3.56
6	8	1.0	39.5	2.85
8	6	1.0	37.9	1.93
10	4	1.0	32.5	1.91

TABLE III. DUPLICATE ANALYSES OF RADIOACTIVE CHOLESTEROL ISOLATED FROM HUMAN PLASMA

ACETATE-1-C^{14} INJECTION (HOURS)	MILLIGRAM PER SAMPLE	TOTAL COUNTS	NET C.P.M.	CHOLESTEROL D/M/MG.	μC PER GRAM
2	1.250	3,510	3,465	2,772	2.45
	1.125	3,454	3,409	3,030	2.68
4	1.000	2,736	2,691	2,691	2.38
	1.175	3,443	3,398	2,892	2.55
6	0.600	1,671	1,626	2,710	2.39
	1.450	3,540	3,495	2,410	2.13
24	1.525	2,325	2,280	1,495	1.32
	0.775	1,129	1,074	1,386	1.22

Background = 55 c.p.m.
Carbon-14 Efficiency = 50.9 per cent.

TABLE IV. PRECISION AND ACCURACY OF ISOLATING AND COUNTING RADIOACTIVE CHOLESTEROL

NUCLIDE	MILLIGRAM PER SAMPLE	TOTAL COUNTS	NET C.P.M.	CHOLESTEROL D/M/MG.	RECOVERY (PER CENT)
Carbon-14*	0.436	1,391	100	524	109
	0.450	1,349	96	443	92
	0.450	1,370	98	499	104
	0.460	1,424	103	513	107
	0.460	1,419	103	513	107
				S. D. = 8.8 per cent	
Tritium†	0.440	1,713	40	1,610	105
	0.480	1,631	36	1,491	97
	0.500	1,594	34	1,332	87
	0.480	1,713	40	1,630	107
	0.490	1,674	39	1,750	114
				S. D. = 10 per cent	

Background = 39 c.p.m.* and 46 c.p.m.†
Samples all counted to a standard error of less than 5 per cent.

Measurement of the Radioactive Digitonide.—The use of the liquid scintillation counter for estimating radioactivity of weak β-emitting nuclides has two advantages. Because of its geometry, no self-absorption correction factors are necessary and steroids samples containing tritium and/or carbon-14 could be counted in the same sample.[7] The question of solubilizing the digitonide in a counting system of practical efficiency was accomplished by using dioxane, ethanol, and toluene. The effect of acid and dioxane concentration is shown in Tables I and II. Routinely, the second aliquot of dioxane (usually 1 or 2 ml.) was added to a 5-dram bottle containing 30 mg. of scintillator, 2,5-diphenyloxozole, one milliliter of absolute ethanol, and sufficient toluene to make a final sample volume of 15 ml. The quenching effect of the digitonide (1.0 to 10.0 mg.) in this counting system was checked and found to be absent. The reproducibility and accuracy as well as the precision of isolating radioactive cholesterol is given in Tables III and IV.

DISCUSSION

The procedure described in this paper makes it possible to determine simultaneously the quantitative amount as well as the radioactivity of a micro cholesterol sample. Studies with known concentrations of sterol gave recoveries of 92 per cent with a standard deviation of 4.2 per cent. The final procedure, applicable to samples containing as little as 100 gamma of cholesterol, was found to be quick, convenient, and accurate. The method suffers from the drawback that the steroid isolated by the digitonin method and measured by the Liebermann-Burchard reaction is not completely specific for cholesterol. These two important factors should be kept in mind as qualifying any interpretation of data obtained by this method using labeled precursors of cholesterol.[12, 13] Following the administration or use of the labeled product, *the specific activity of the isolated sterol is not seriously affected* because the possible contaminants are essentially nonradioactive and therefore do not influence the interpretation of the experiment to the same degree as would radioactive impurities.

SUMMARY

1. A quick, convenient, and quantitative micromethod for isolating, measuring, and assaying radioactive free and ester cholesterol from plasma or tissue has been presented.

2. A solvent system applicable for assaying cholesterol digitonide in a liquid scintillation counter has been devised.

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MICROMETHOD FOR THE DIRECT DETERMINATION OF SERUM TRIGLYCERIDES

E. Van Handel, Ph.D., and D. B. Zilversmit, Ph.D.
Memphis, Tenn.
With the Technical Assistance of Kirk Bowman, B.S.

INTRODUCTION

A WEALTH of information is available on the concentration of plasma cholesterol and phospholipids. However, few investigators have attempted to survey the variations of blood triglycerides in health and disease. This paucity of data is probably the result of the inadequacy of current analytical methods. For example, the solvents used for the extraction of lipids dissolve appreciable amounts of nonlipid material and extensive purification or re-extraction is required prior to the determination of total lipids by oxidative or gravimetrical methods. Furthermore, lipid-like materials such as free fatty acids, steroids, mono-, and diglycerides would be included in these nonspecific total lipid estimations. With the exception of the newer, more elaborate, chromatographic techniques, all routine determinations of triglycerides are based on the estimation of the difference between the total lipids and the sum of cholesterol, cholesterol ester, and phospholipid. As normally the triglycerides constitute only a small fraction of the total lipids, the need for a more specific method seems obvious. The present publication presents a micromethod for the direct determination of plasma, serum, or whole blood triglycerides, which is based on the quantitative measurement of the glycerol moiety of these molecules.

MATERIALS AND METHODS

Reagents.—

Fat solvents: Redistilled technical grade chloroform is satisfactory. Keep in dark bottle.

*Doucil**: Activate by heating for 4 hours at 125° C. Keep in closed bottle.

Alcoholic KOH (0.4 per cent): Dissolve 2 Gm. of reagent grade KOH in 95 per cent redistilled ethyl alcohol and dilute to 100 ml. Dilute 10 ml. stock to 50 ml. with 95 per cent alcohol on the day of use.

Sulfuric acid (0.2 N): Dilute 3 ml. of concentrated H_2SO_4 to 500 ml. with water.

Sodium metaperiodate (0.05 M): Dissolve 1.07 Gm. of $NaIO_4$ in 100 ml. of water.

Sodium arsenite (0.5 M): Dissolve 2.25 Gm. of NaOH and 5 Gm. reagent grade arsenic trioxide and dilute to 100 ml. with water.

From the Division of Physiology, University of Tennessee, Memphis, Tenn.

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*Doucil is a zeolite obtained from W. A. Taylor Company, 7300 York Road, Baltimore, Md.

Chromotropic acid (0.2 per cent): Dissolve 2 Gm. of chromotropic acid* (or 2.24 Gm. of the sodium salt) in 200 ml. of water. Separately add 600 ml. of concentrated H_2SO_4 to 300 ml. of water, cooled in ice. When cool, add the diluted H_2SO_4 to the chromotropic acid solution. Store in brown bottle and prepare fresh every 2 to 3 weeks.

Triglyceride standard solution (0.05 mg./ml.): Dissolve 0.5 Gm. of commercial corn oil in chloroform and make to 100 ml. in a glass-stoppered volumetric flask. Dilute working standard 1 to 100 with chloroform. It is advisable to check the concentrated corn oil solution now and then gravimetrically to guard against increases in concentration by evaporation.

PROCEDURE

Place 4 Gm. of Doucil in a 28 × 130 mm. glass-stoppered tube, add 2 ml. of chloroform, and shake. Place 1 ml. of plasma on top of the Doucil, mix thoroughly, and add 18 ml. of chloroform. Stopper the tube and shake intermittently but vigorously for about 10 minutes. Filter through a coarse fat-free paper and pipette 1 to 3 ml. portions of the supernatant, depending on the amount of triglyceride present, into 3 glass-stoppered or screw-capped glass tubes (15 × 130 mm.). At this point, pipette three 1-ml. portions of the standard corn oil solution into glass tubes and evaporate the solvent from all tubes. To 2 out of each 3 standards and unknowns add 0.5 ml. of alcoholic KOH (saponified sample) but to the third aliquot add 0.5 ml. of alcohol (unsaponified sample). Keep all tubes at 60 to 70° C. for 15 minutes. Add 0.5 ml. of 0.2N H_2SO_4 and place the tubes in a gently boiling water bath† for about 15 minutes to remove the alcohol. After cooling, the glycerol content is determined essentially according to the procedure of Lambert and Neish.[1] Add 0.1 ml. of periodate solution from a graduated pipette. After 10 minutes, the oxidation is stopped by the addition of 0.1 ml. of sodium arsenite. A yellow color of iodine appears and vanishes in a few minutes. Then add 5 ml. chromotropic acid reagent and mix. Heat for one-half hour at 100° C. in the absence of excessive light.‡[1] After cooling, determine the optical density at 570 mμ. The color remains stable for several hours.

CALCULATION OF RESULTS

$$\frac{\text{O.D. saponified unknown—O.D. unsaponified unknown}}{\text{O.D. saponified standard—O.D. unsaponified standard}} \times 0.05 \times \frac{2{,}000}{\text{ml. of chloroform extract}} = \text{mg. per cent triglyceride.}$$

To convert mg. per cent to millimoles per liter, multiply by 0.0114.

RESULTS

Standard Curve.—Fig. 1 shows the relationship between the optical density and the amounts of corn oil and different aliquots of a chloroform extract of plasma. Thus, a good linearity is established from 0 to 200 gammas of triglyceride. In several experiments, the recovery of glycerol in a known amount of oil was checked against a glycerol standard which was prepared by diluting

TABLE I. GLYCEROL CONTENT OF DIFFERENT FATS (PER CENT)

	THEORETICAL	EXPERIMENTAL
Cocoa butter	10.6	10.9
Coconut oil	13.4	13.7
Corn oil	10.5	10.3
Cottonseed oil	10.8	10.5
Tripalmitin (Eastman)	11.4	10.5

*Chromotropic acid is 4,5-dihydroxy-2,7-naphthalenedisulfonic acid.

†It is advisable to keep the water level of the bath only slightly above the surface of the reaction mixture in order to avoid evaporation of the water from the tubes.

‡Tubes may be heated in a boiling water or steam bath but should then be stoppered to exclude water vapors from the reaction mixture. We preferred to heat the tubes in a thermostatically controlled heating block (Biochemical Associates, Oakland, Calif.).

analytic reagent grade glycerol assayed by refractometry for water content. The glycerol content of five different fats are listed in Table I together with the theoretical amounts calculated from the average molecular weight of the fatty acids.[2] The experimental results are in good agreement with the calculated ones.

Extraction of Triglycerides From Plasma.—The extraction of lipids from plasma with the aid of Doucil and chloroform was first proposed by Forbes.[3] For the present method it became necessary to establish that complete extraction of triglycerides could be achieved without dissolving the plasma phospholipids.

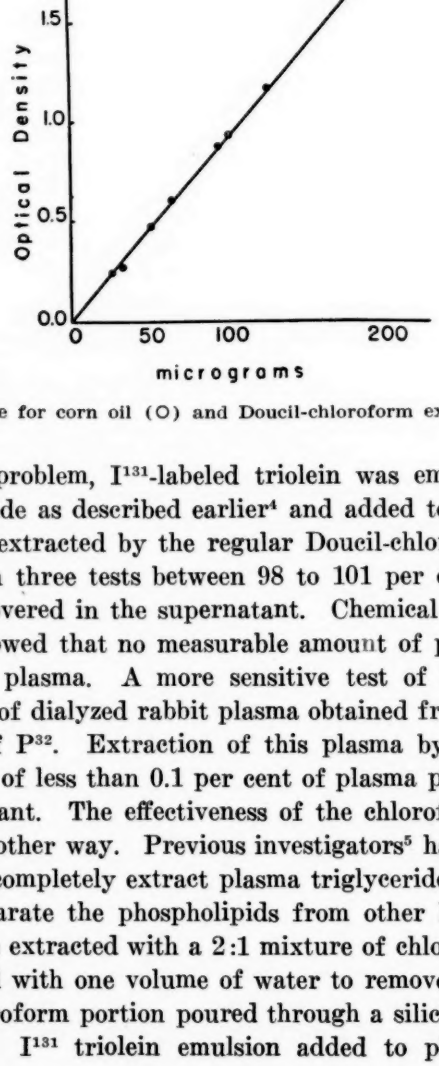

Fig. 1.—Calibration curve for corn oil (○) and Doucil-chloroform extract of dog plasma (●).

To investigate this problem, I^{131}-labeled triolein was emulsified with a small amount of phosphatide as described earlier[4] and added to plasma. An aliquot of plasma was then extracted by the regular Doucil-chloroform procedure and it was found that in three tests between 98 to 101 per cent of the added I^{131} triglyceride was recovered in the supernatant. Chemical analysis for phospholipid phosphorus showed that no measurable amount of phospholipid had been extracted from the plasma. A more sensitive test of this latter point was provided by the use of dialyzed rabbit plasma obtained from an animal injected with a large dose of P^{32}. Extraction of this plasma by the above procedure showed the presence of less than 0.1 per cent of plasma phospholipid P^{32} in the chloroform supernatant. The effectiveness of the chloroform-Doucil procedure was tested in yet another way. Previous investigators[5] have shown that chloroform-methanol will completely extract plasma triglycerides and that silicic acid may be used to separate the phospholipids from other lipids.[6] Thus, several plasma samples were extracted with a 2:1 mixture of chloroform-methanol. The extract was inverted with one volume of water to remove the methanol, centrifuged, and the chloroform portion poured through a silicic acid column according to Borgström.[6] I^{131} triolein emulsion added to plasma was completely

recovered by the silicic acid method. Triplicate analyses of a serum sample by the Doucil method and by the chloroform-methanol silicic acid method showed good agreement between the two procedures (Table II).

Triglyceride Concentrations in Plasma of Different Species.—Plasma triglycerides determined on a group of 14 young men in the postabsorptive state are shown in Table III. The mean value of 78 mg. per cent agrees well with the value of 90 mg. per cent given by Peters and Man.[7] The values for dogs were obtained on mongrels bled after a 16-hour fast; the rabbits, on the other hand, had access to rabbit chow.

TABLE II. COMPARISON OF DOG PLASMA TRIGLYCERIDES DETERMINED BY DOUCIL AND BY SILICIC ACID METHODS IN MILLIGRAM PER CENT

ALIQUOT	DOUCIL	SILICIC ACID
1	33.8	34.5
2	33.7	35.5
3	33.5	35.2

TABLE III. PLASMA TRIGLYCERIDES (MILLIGRAM PER CENT)

	MEAN	RANGE	NUMBER OF CASES	STANDARD ERROR*
Normal men (postabsorptive)	79	37-134	14	7.1
Dogs (postabsorptive)	54	26-88	8	7.0
Rabbits (fed)	95	40-162	20	8.8

$$*\text{S.E.} = \sqrt{\frac{\Sigma X^2}{N(N-1)} - \frac{(\Sigma X)^2}{N^2(N-1)}}$$

DISCUSSION

The procedure outlined in the previous section consists essentially of four steps: the selective quantitative extraction of triglycerides from plasma, the complete saponification of the triglycerides, the oxidation of glycerol to formaldehyde and formic acid, and the colorimetric estimation of formaldehyde. To test the quantitative aspects of the procedure, each step had to be checked independently. Furthermore, it appeared that several alternatives were possible and the final choice of the method depends somewhat on the particular facilities available in any given laboratory. Therefore, it would seem useful to discuss briefly some additions to and possible modifications of the procedure.

The extraction of triglycerides from plasma with the aid of Doucil gives a chloroform supernatant containing not only the total amount of plasma triglyceride but the cholesterol and cholesterol ester as well. Thus, if one is interested in obtaining a quantitative estimate of plasma cholesterol in addition to the triglyceride one may use the chloroform supernatant and add to it directly acetic anhydride and sulfuric acid for the colorimetric estimation of total cholesterol.[3] If, however, one desires to determine the phospholipid in addition to cholesterol and triglyceride, an alternative procedure is suggested. One milliliter of plasma is extracted with 2:1 chloroform-methanol in a 25-ml. volumetric flask, the extract is filtered, and a 20-ml. aliquot of the filtrate is carefully inverted with an equal volume of water and centrifuged. This gives a two-phase system consisting of a solution of lipids primarily in chloroform

and an aqueous-methanol supernatant. The aqueous phase is removed as quantitatively as possible by means of a syringe pipette and the chloroform layer which may still contain a little supernatant on top is poured through an activated silicic acid column.* The column is then washed with two subsequent portions of about 5 ml. of chloroform each and the eluate which contains all the triglyceride, cholesterol, and cholesterol ester is made to 25 ml. with chloroform. The phospholipid stays on the column and may be determined by estimation of the phosphorus content of a methanol eluate.[6] The procedure described here for plasma may also be applied to whole blood or to chloroform-methanol extracts of tissues. For whole blood, the Doucil procedure may also be used with good results.[8] Extraction of added I^{131} triglyceride emulsion from whole blood with chloroform in the presence of Doucil was quantitative and a comparison between the triglyceride content of whole blood as determined by the Doucil procedure and the silicic acid column gave agreement which was as good as that described for plasma.

The proposed saponification procedure was arrived at by the following experiments: saponification of small amounts of triglycerides with aqueous alkali of various concentrations led to unsatisfactory recovery of glycerol; saponification with alcoholic KOH with subsequent evaporation of alcohol led to considerable loss of glycerol. Indeed, when alcohol was distilled from an alcoholic glycerol solution in a boiling water bath, considerable amounts of glycerol appeared in the distillate. Such loss of glycerol was not observed when the alcoholic glycerol solution was diluted with an equal volume of water. Failure to remove the ethanol before oxidation with periodate produced a turbidity during the heating with the chromotropic acid. The development of this turbidity seems to be related to the presence of cholesterol since it did not occur in standard corn oil solutions to which no cholesterol had been added. To avoid these difficulties, saponification was carried out in 95 per cent ethanol below the boiling point of alcohol. A 15-minute heating between 60° and 70° C. appeared to be satisfactory since no difference in glycerol recovery was observed when corn oil was saponified at 65° C. from 5 to 60 minutes.

The determination of the presaponification blank (which includes the reagent blank) increases the specificity of the method and prevents errors from the possible presence of formaldehydogenic steroids or monoglycerides in the lipid extract. Normally the presaponification blank does not exhibit the typical formaldehyde chromotropic-acid color. Thus, one may easily distinguish between this nonspecific color of the blank and the presence of formaldehydogenic materials in the lipid extract. At this point, it should be noted that the presence of α-monoglyceride in the lipid extract would produce a presaponification color which is half that produced after saponification, whereas the presence of β-monoglycerides and diglycerides would not be detected in this manner. So far, we have not found any instances where the color in the unsaponified samples of blood or plasma resembled that produced by glycerol.

*A convenient way of preparing silicic acid columns for serial determinations is the use of 10-ml. syringe barrels in which a small plug of glass wool is inserted, followed by a 1-Gm. portion of 1:1 mixture of activated silicic acid (Mallinckrodt) and Supercel (Johns-Manville).

SUMMARY

A microprocedure for the direct determination of triglyceride concentrations in biologic specimens is presented. The method depends on the quantitative removal of phosphatides from the sample and the subsequent determination of esterified glycerol. The procedure has been tested on whole blood and plasma.

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A TECHNIQUE FOR THE SAFE HANDLING OF COCCIDIOIDES IMMITIS CULTURES

MILTON HUPPERT, PH.D.
SAN FERNANDO, CALIF.

INTRODUCTION

FOR at least ten years there have been repeated warnings of the danger to laboratory personnel in the handling of cultures of *Coccidioides immitis*.[1-7] Smith and Harrell[2] reported 6 cases involving laboratory workers as a consequence of accidental contamination of the outside of flasks with liquid cultures of this fungus. Looney and Stein[3] reviewed 33 cases of accidental infections described in the literature and reported one new case of disseminated infection in a laboratory worker. Sulkin and Pike[4] analyzed a total of 1,342 laboratory infections, of which 63 were caused by fungi, principally *C. immitis*.

Despite the hazards involved, there are occasions when many laboratories must handle cultures of *C. immitis*. These instances are no longer restricted to the institutions located in the recognized endemic areas but may occur in any geographical location, since individuals who have contracted the infection may not exhibit disease until after they have left the endemic region.[3, 8-13] Evans and his associates[13] warned that coccidioidomycosis may be encountered by physicians in any part of the United States. While all microbiological laboratories must be aware of the dangers inherent in the handling of cultures of *C. immitis*, this is particularly true for those clinical laboratories where this fungus has been encountered only infrequently or not at all. It is in these laboratories where inexperience with *C. immitis* is most likely to be associated with lack of adequate facilities for handling such cultures safely.

It is the purpose of this report to describe a technique which will enable any laboratory to isolate and identify *C. immitis* with safety.

MATERIALS AND METHODS

All strains of *C. immitis* used were obtained from the stock culture collection of the Mycology Research Laboratory, San Fernando Veterans Administration Hospital. These strains were selected as representative of a range of characteristics, such as, recently isolated cultures and those which had been subcultured for varying periods, the presence or absence of arthrospores, and the presence or absence of aerial hyphae. For preliminary testing a strain of Penicillium was used.

Four media were used: Sabouraud's dextrose agar; Sabouraud's dextrose agar with added antibiotics; Blood Agar Base (Difco); Blood Agar Base with added antibiotics. The antibiotics were sterilized separately by filtration and incorporated into the sterile

From the Mycology Research Laboratory, Veterans Administration Hospital, San Fernando, Calif.

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media after they had been melted and cooled to 50° C. The concentrations used were: penicillin, 20 units per milliliter; streptomycin, 40 units per milliliter; cycloheximide, 0.5 mg. per milliliter.[14]

After adding the antibiotics, the media were poured into sterile 2-ounce prescription bottles, each fitted with a screw cap containing a rubber diaphragm (Fig. 1). The bottles were slanted and the medium allowed to harden. When ready for inoculation, the bottles and the stock cultures of *C. immitis* were placed in a transfer chamber which was then sealed. The front of this chamber contained two rubber gloves with an attached accordion-type sleeve to permit relatively free arm movement within the chamber. All culture transfers were made in this manner. Air was circulated through the chamber by means of an exhaust fan. Both incoming and outgoing air were filtered, and a constant cultural check was maintained on the exhaust air to determine whether or not spores passed the filter.

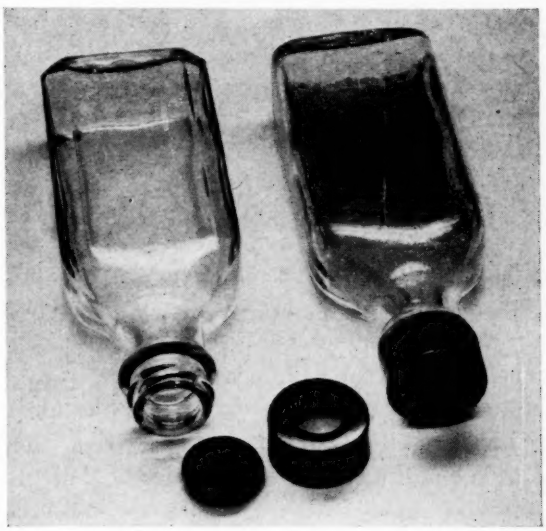

Fig. 1.—Two culture bottles with rubber-diaphragm screw caps are shown. One is empty and disassembled; the other contains agar and is assembled ready for use.

Fluid was added to or withdrawn from the culture bottles with Luer-Lok needles and syringes. The rubber diaphragm was painted with 3 per cent tincture of iodine before insertion of the needle. Sterile cotton, soaked in 70 per cent aqueous ethanol, was wrapped around the needle and the point of penetration in the rubber diaphragm. After fluid was drawn into the syringe, the needle and syringe were extracted very slowly, and, after the needle point had cleared the diaphragm, it was held tightly within the alcohol-soaked cotton to prevent any aerosol dispersal of infectious material into the surrounding air.

Petri plates containing Sabouraud's dextrose agar were exposed continuously throughout all experiments. These culture dishes were placed both in the transfer chamber and in the laboratory.

All techniques were done first with the strain of Penicillium and then repeated with the *C. immitis* cultures.

RESULTS

Testing for Disposal of Fungous Spores During Transfer Operations.—Using the Penicillium strain 5 transfers were made from a 2-week-old stock culture to fresh media. During the operation 6 plates of media were exposed

inside the chamber, and the exhaust air from the chamber was allowed to impinge on an additional plate. After completion of the transfers, the exposed plates were incubated at 30° C. for 2 weeks. The experiment was repeated 10 times.

TABLE I. THE DISPERSION OF PENICILLIUM SPORES DURING TRANSFERS FROM STOCK CULTURE TO FRESH MEDIA USING A THIN TRANSFER NEEDLE

EXPERIMENT	COLONIES OF PENICILLIUM RECOVERED	
	INSIDE CHAMBER	FROM CHAMBER EXHAUST
1	6	0
2	3	0
3	0	0
4	5	0
5	2	0
6	0	0
7	1	0
8	1	0
9	4	0
10	7	0
	29	0

As recorded in Table I, a total of 29 colonies of Penicillium were recovered from plates exposed inside the chamber but none was found on plates exposed to the chamber exhaust, indicating efficient removal of spores from the air stream by the filter. It had been noted during the transfer operations that occasionally the thin wire needle seemed to "whip" slightly as it was withdrawn after cutting out a piece of agar containing growth. It was suspected that this could result in dispersing some spores into the air. The experiment was repeated, therefore, using a stiff dissecting needle, and the results are recorded in Table II. While the number of colonies of Penicillium was reduced considerably, it should be noted that, even with the most careful technique, some spores still escape into the environment.

TABLE II. THE DISPERSION OF PENICILLIUM SPORES DURING TRANSFERS FROM STOCK CULTURE TO FRESH MEDIA USING A STIFF DISSECTING NEEDLE

EXPERIMENT	COLONIES OF PENICILLIUM RECOVERED	
	INSIDE CHAMBER	FROM CHAMBER EXHAUST
1	0	0
2	0	0
3	1	0
4	0	0
5	2	0
6	0	0
7	0	0
8	0	0
9	1	0
10	1	0
	5	0

Sterilization of the Chamber.—Two sets of 10 cultures each were inoculated with Penicillium in the transfer chamber. On one set the caps were screwed down tightly while on the other set the caps were loose. Approximately 10

ml. of 37 per cent formaldehyde were poured into a beaker. After 24-hour exposure to formaldehyde vapor, all cultures were removed and incubated at 30° C. for 2 weeks. The experiment was repeated 10 times.

In the bottles which had been capped tightly, the Penicillium grew abundantly, while there was no growth in the bottles with loose caps. The same results were obtained when the experiment was repeated with strains of *C. immitis.*

In view of the results obtained from these two sets of experiments, it was felt that further work with cultures of *C. immitis* could be accomplished without endangering laboratory personnel.

Handling of Cultures of C. immitis.—Ten cultures of *C. immitis* were made on each of the four media. The bottles were capped tightly and exposed to formaldehyde for 24 hours. All 40 cultures were incubated at 30° C. for 2 weeks. Excellent growth was obtained in each culture.

Varying amounts of three concentrations of formaldehyde were injected through the rubber diaphragm of the tightly closed caps of 9 cultures of each of the 4 sets of media. One bottle of each medium was kept as a control. After 24 and 48 hours at 37° C., transfers were made from each culture to fresh media and incubated at 30° C. for one month. The results are recorded in Table III. All nonexposed control cultures were positive. All subculture bottles which were negative were reinoculated at the end of the experiment with a viable culture and growth occurred in each case.

TABLE III. GROWTH OF SUBCULTURES OF C. IMMITIS (C-25) AFTER EXPOSURE TO VARYING AMOUNTS OF FORMALDEHYDE FOR 24 AND 48 HOURS

GROWTH ON	PER CENT CONCENTRATION OF FORMALDEHYDE	GROWTH ON SUBCULTURE					
		AFTER 24 HOUR EXPOSURE			AFTER 48 HOUR EXPOSURE		
		VOL. FORMALDEHYDE ADDED			VOL. FORMALDEHYDE ADDED		
		0.5 ML.	2.0 ML.	8.0 ML.	0.5 ML.	2.0 ML.	8.0 ML.
Sabouraud's Dextrose Agar (SDA)	0.5	+	−	−	+	−	−
	2.0	−	−	−	−	−	−
	8.0	−	−	−	−	−	−
SDA with Antibiotics	0.5	+	+	−	+	−	−
	2.0	−	−	−	−	−	−
	8.0	−	−	−	−	−	−
Blood Agar Base (BAB)	0.5	+	−	−	+	+	−
	2.0	−	−	−	−	−	−
	8.0	−	−	−	−	−	−
BAB with Antibiotics	0.5	+	+	−	+	−	−
	2.0	−	−	−	−	−	−
	8.0	−	−	−	−	−	−

This experiment was repeated using 10 strains of *C. immitis*, 0.5 ml. amounts of the 3 concentrations of formaldehyde, and Sabouraud's dextrose agar. All cultures were done in triplicate. Representative results are recorded in Table IV. All cultures exposed to 8 per cent formaldehyde were sterilized, while the results were variable with the 0.5 and 2 per cent concentrations.

TABLE IV. GROWTH OF SUBCULTURES OF TEN STRAINS OF C. IMMITIS AFTER EXPOSURE TO 0.5 ML. OF VARYING CONCENTRATIONS OF FORMALDEHYDE FOR 24 AND 48 HOURS

PER CENT CONCENTRATION OF FORMALDEHYDE	GROWTH ON SUBCULTURE	
	AFTER 24 HOUR EXPOSURE	AFTER 48 HOUR EXPOSURE
0.5	26/30*	9/30
2.0	2/30	0/30
8.0	0/30	0/30

*Numerator represents the total number of subcultures which were positive for *C. immitis* and the denominator the total number of subcultures made.

A single culture of each of the 10 strains was made on Sabouraud's dextrose agar and on Blood Agar Base. After incubation at 30° C. for 2 weeks, slides were made from each culture in 20 per cent NaOH in glycerin-water (50:50). The cultures were killed by exposure to 0.5 ml. of 8 per cent formaldehyde for 24 hours and a second set of slides was made. The microscopic appearance of a culture which had been killed by formaldehyde was comparable to that of the corresponding viable culture.

Suspensions of viable material were made from cultures grown in the bottles with rubber-diaphragm screw caps. Five milliliters of saline were injected through the diaphragm after the cap was closed tightly. The bottle was shaken vigorously and the resulting suspension was withdrawn with a Luer-Lok needle and syringe. Extreme care was taken to prevent the escape of an aerosol, particularly at the moment the needle was withdrawn from the diaphragm. No colonies of *C. immitis* were detected on plates exposed in the area during the operation. No difficulty was encountered in obtaining suspensions from those cultures which produced aerial hyphae. Frequently, however, macroscopically visible particles would separate from the agar and from the colony itself. These could be avoided by inverting the culture, allowing the particles to settle, and inserting the syringe needle to a level above the sediment before withdrawing any fluid. Suspensions with one million or more cells per milliliter could be prepared without ever opening the culture. Such suspensions were satisfactory for injecting animals to produce coccidioidomycosis as the final step in the identification of *C. immitis*.

DISCUSSION

Since it is quite possible that any clinical laboratory might isolate a culture of *C. immitis* at some time, it is imperative that each laboratory have the facilities available to handle such cultures with safety for the personnel involved. Not many laboratories are equipped with completely sealed transfer chambers for this purpose, and it would be impractical for them to maintain such an apparatus on the chance that it would be used only rarely. This disadvantage can be overcome, however, by using an inexpensive bottle fitted with a rubber-diaphragm screw cap for culturing specimens which might contain *C. immitis*. As a general rule, these bottles could be used for culturing any specimen which might contain microorganisms capable of producing pulmonary disease in man. The greatest danger, however, lies in the possibility

of accidentally breaking the container of such cultures. The 2-ounce prescription bottles used here are quite sturdy and have remained undamaged after dropping to a concrete floor. Nevertheless, an unbreakable plastic container, which could be sterilized, would be even better.

The following procedure is suggested specifically for specimens in which *C. immitis* may be encountered. Portions of the specimen should be streaked on several media in rubber-diaphragm screw cap bottles. If, after incubation, there should be any colonies suspected of being *C. immitis,* then approximately 5 ml. of sterile saline should be injected through the rubber diaphragm while keeping alcohol-soaked cotton wrapped around the needle and the bottle cap. If the culture is contaminated with other microorganisms, penicillin and streptomycin should be added to the saline. With the alcohol-soaked cotton held around the cap, the bottle should be shaken vigorously. The suspension can be withdrawn being careful to hold the wad of cotton tightly around the syringe needle which is removed very slowly. Five-tenths milliliter of the suspension can be injected, intraperitoneally, into each of several mice, and the remainder can be injected through the rubber diaphragm of a bottle of fresh media. The original culture can be sterilized by injecting formaldehyde (0.5 ml. of 8 per cent, or greater, concentration) and incubating for 24 hours at 37° C., at which time the bottle can be opened safely for making slide preparations.

SUMMARY

A technique is described for handling cultures of *Coccidioides immitis* with safety. This consists of growing the fungus on media in bottles fitted with a rubber-diaphragm screw cap.

Suspensions of fungous spores can be made by injecting fluid through the rubber diaphragm with a needle and syringe, shaking the bottle, and then withdrawing the suspension with a needle and syringe. As a precaution against the formation of aerosols of infectious material, alcohol-soaked cotton should be wrapped around the screw cap and the syringe needle during the process.

Cultures in these bottles can be sterilized by the injection of 0.5 ml. of at least 8 per cent formaldehyde followed by incubation at 37° C. After 24 hours the bottles can be opened for making slide preparations. The microscopic morphology of formaldehyde killed cultures is comparable to that of the viable culture.

ADDENDUM

After the present manuscript had been accepted for publication, it was brought to the attention of the author that the bottle pictured in Fig. 1 is no longer manufactured. Therefore, the technique has been revised to adapt readily available bottles for this use. The screw caps from prescription bottles are soaked in detergent solution until the liners can be removed. A hole, one-eighth inch in diameter, is drilled through the center of each cap. Suitable rubber diaphragms are fixed to the inside of the caps with rubber cement and the caps replaced on the bottles.

It has been found advantageous to insert a cotton-plugged, sterile, syringe needle through the diaphragm during incubation of cultures. Strains of *Coccidioides immitis* develop aerial mycelium with arthrospores much more profusely under these conditions than when the needle is omitted.

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